Activity 7.7. Evaluating a diallel assay in Villavicencio

Specific objectives

1. To evaluate a diallel study for resistance to CBB and SED

Methodology

A 10 × 10 diallel study, comprising 45 families with 30 plants each, was evaluated for the plants' reaction to CBB and SED under natural disease pressure and according to a disease severity scale of 1 to 5, where 1 = no symptoms and 5 = plant death.

The diallel was planted with three replicates at two sites at CORPOICA's "La Libertad" station. Only one site had disease pressure, so the other was not evaluated for disease. The 10 genotypes conforming the diallel were:

CM 4574-7	CM 6740-7	CM 7033-3	SM 1219-9	SM 1565-15
SM 2058-2	SM 2219-11	HMC-1	M Per 183	M Tai 8

Results

The severity average for the families oscillated between 2.1 and 3.2 for CBB and between 2.2 and 4.2 for SED. Greater severity and variation among plants in each family was observed for SED than for CBB (Table 7.11), probably because CBB pressure was not high.

Table 7.12 presents averages for CBB and SED severity for all progenies obtained from each parent (810 individuals: 9 crosses, 30 plants/cross, 3 replicates). According to the data, CM 4574-7, SM 1565-15, and CM 7033-3 had progenies with the greatest resistance to SED, whereas the CM 4574-7 and M Tai 8 progenies had the highest CBB resistance. CM 4574-7 tended to have the highest general combining ability for resistance to these diseases. M Per 183, HMC-1, M Tai 8, and CM 6740-7 had progenies with the lowest resistance.

Table 7.11. Range, average, and standard deviation (SD) of severity[a] of cassava bacterial blight (CBB) and superelongation disease (SED) for each diallel crossing in cassava, Villavicencio, Colombia.

Cross[b]	Statistic	CBB	SED	Cross[b]	Statistic	CBB	SED	Cross[b]	Statistic	CBB	SED
1 × 2	Range	2.0-3.5	1.0-4.0	2 x 9	Range	2.0 - 4.0	2.0 - 4.5	5 x 6	Range	2.0 - 4.0	1.0 - 4.5
	Avg	2.5	2.3		Avg	2.7	3.9		Avg	2.8	2.3
	SD	0.3	0.6		St dev	0.6	0.5		St dev	0.5	0.6
1 × 3	Range	2.0-3.5	1.0-4.0	2 x 10	Range	2.0 - 3.5	2.5 - 4.5	5 x 7	Range	2.0 - 3.5	1.0 - 4.0
	Avg	2.5	2.2		Avg	2.5	3.4		Avg	2.5	2.2
	SD	0.3	0.6		St dev	0.4	0.6		St dev	0.3	0.5
1 × 4	Range	2.0-3.5	1.5-5.0	3 x 4	Range	2.0 - 4.0	1.0 - 5.0	5 x 8	Range	2.0 - 4.0	1.5 - 4.0
	Avg	2.7	2.5		Avg	2.6	2.4		Avg	2.8	2.9
	SD	0.3	0.7		St dev	0.5	0.7		St dev	0.5	0.7
1 × 5	Range	2.0-3.5	1.0-4.0	3 x 5	Range	2.0 - 4.0	1.5 - 5.0	5 x 9	Range	2.0 - 4.0	2.0 - 4.5
	Avg	2.5	2.4		Avg	2.6	2.2		Avg	2.7	3.3
	SD	0.3	0.7		St dev	0.5	0.7		St dev	0.5	0.7
1 × 6	Range	2.0-4.5	1.0-4.5	3 x 6	Range	2.0 - 5.0	1.5 - 5.0	5 x 10	Range	2.0 - 3.5	1.5 - 4.5
	Avg	2.5	2.6		Avg	3.2	2.7		Avg	2.4	2.9
	SD	0.5	0.9		St dev	0.9	0.8		St dev	0.4	0.7
1 x 7	Range	2.5 - 3.5	1.5 - 4.0	3 x 7	Range	2.0 - 4.5	1.0 - 4.0	6 x 7	Range	2.0 - 4.0	1.0 - 4.0
	Avg	2.6	2.6		Avg	2.9	2.4		Avg	2.8	2.6
	St dev	0.4	0.7		St dev	0.7	0.7		St dev	0.5	0.6
1 x 8	Range	2.0 - 3.5	1.0 - 4.0	3 x 8	Range	2.0 - 4.0	1.5 - 4.5	6 x 8	Range	2.0 - 4.5	1.5 - 4.0
	Avg	2.6	2.5		Avg	2.9	2.7		Avg	3.2	2.5
	St dev	0.4	0.5		St dev	0.7	0.8		St dev	0.5	0.6
1 x 9	Range	2.0 - 4.0	2.0 - 4.0	3 x 9	Range	2.0 - 4.0	1.5 - 4.5	6 x 9	Range	2.0 - 4.0	2.0 - 4.5
	Avg	2.6	3.2		Avg	2.8	3.5		Avg	2.8	3.5
	St dev	0.3	0.6		St dev	0.6	0.6		St dev	0.6	0.6
1 x 10	Range	2.0 - 4.0	1.5 - 5.0	3 x 10	Range	2.0 - 4.0	1.5 - 4.5	6 x 10	Range	2.0 - 4.0	1.0 - 4.5
	Avg	2.3	3.0		Avg	2.7	2.8		Avg	2.6	3.1
	St dev	0.4	0.8		St dev	0.6	0.7		St dev	0.6	0.8
2 x 3	Range	2.0 - 4.0	1.5 - 4.5	4 x 5	Range	2.0 - 4.0	2.0 - 4.5	7 x 8	Range	2.0 - 4.5	1.5 - 4.5
	Avg	2.8	3.0		Avg	2.6	3.0		Avg	2.8	2.7
	St dev	0.6	0.8		St dev	0.5	0.6		St dev	0.6	0.7
2 x 4	Range	2.0 - 4.0	2.0 - 5.0	4 x 6	Range	2.0 - 4.0	1.5 - 4.5	7 x 9	Range	2.0 - 4.0	2.0 - 4.5
	Avg	2.5	3.3		Avg	3.0	3.1		Avg	2.6	3.5
	St dev	0.5	0.7		St dev	0.7	0.6		St dev	0.6	0.6

2 x 5	Range	2.0 - 4.0	1.0 - 4.5	4 x 7	Range	2.0 - 4.0	1.5 - 4.0	7 x 10	Range	2.0 - 4.0	1.0 - 4.0
	Avg	2.5	2.5		Avg	2.6	2.6		Avg	2.7	3.1
	St dev	0.4	0.7		St dev	0.4	0.6		St dev	0.6	0.7
2 x 6	Range	2.0 - 4.5	1.0 - 4.0	4 x 8	Range	2.0 - 3.5	1.0 - 4.0	8 x 9	Range	2.0 - 4.0	3.0 - 5.0
	Avg	3.0	2.9		Avg	2.6	2.8		Avg	2.5	4.2
	St dev	0.7	0.8		St dev	0.4	0.7		St dev	0.6	0.4
2 x 7	Range	2.0 - 4.0	1.5 - 4.5	4 x 9	Range	2.0 - 4.0	2.0 - 4.0	8 x 10	Range	2.0 - 3.5	1.5 - 4.5
	Avg	2.7	3.1		Avg	2.9	3.5		Avg	2.7	3.7
	St dev	0.4	0.7		St dev	0.6	0.5		St dev	0.5	0.6
2 x 8	Range	2.0 - 4.5	1.5 - 5.0	4 x 10	Range	2.0 - 3.5	1.0 - 4.0	9 x 10	Range	2.0 - 3.5	3.0 - 4.5
	Avg	2.9	3.5		Avg	2.5	3.2		Avg	2.1	4.1
	St dev	0.7	0.8		St dev	0.5	0.8		St dev	0.3	0.3

[a]Severity on a scale from 1 to 5, where 1 = no symptoms and 5 = plant death.
[b]Parents of each cross are identified in Table 7.12.

Table 7.12. Disease severity average for progenies of each parent in a dialed study of cassava, Villavicencio, Colombia.

Parent		CBB[a]	SED[b]
Code no.	Genotype		
1	CM 4574-7	2.50	2.54
2	CM 6740-7	2.66	3.09
3	CM 7033-3	2.76	2.66
4	SM 1219-9	2.63	2.92
5	SM 1565-15	2.60	2.61
6	SM 2058-2	2.86	2.84
7	SM 2219-11	2.68	2.76
8	HMC-1	2.75	3.06
9	M Per 183	2.63	3.63
10	M Tai 8	2.50	3.25

a. CBB = cassava bacterial blight.
b. SED = superelongation disease.

Activity 7.8. Evaluating probes of resistance gene analogs in cassava parentals of different crosses for resistance to Phytophthora root rot

Specific objective

1. *To develop molecular markers associated with genes involved in resistance to root rots.*

Methodology

DNA was extracted from leaf tissues of five cassava parental genotypes—M Nga 2, CM 2177-2, M Bra 1045, M CR 81, and M CR 54—using the Gilbertson-Dellaporta protocol (Dellaporta et al. 1983). M Nga 2 is intermediately resistant to *Phytophthora tropicalis* and susceptible to *Phytophthora* isolate MTR6, whereas CM 2177-2 is susceptible to *P. tropicalis* and resistant to isolate MTR6. Genomic restriction with the enzymes *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Dra*I, and *Taq*I was done after gel depurination and denaturation. The digested DNA was transferred overnight to a Hybond N+ membrane, using 10X SSC (NaCl and trisodic citric acid) as transferring solution. The DNA was fixed on the membrane by ultraviolet light in a Stratalinker.

Cells of *Escherichia coli*, strain DH5-α, were transformed by electroporation, introducing pGEM-T Plasmid Vector System (Promega), containing 10 disease resistance gene analogs (RGAs) isolated from maize and rice. Transformed cells were kept at –80°C in glycerol 30%. Minipreparations were prepared with Concert Rapid Plasmid Purification Systems (Gibco-BRL) from transformed cells. A PCR, using primer pairs T7/SP6, M13F/M13R, and T3/T7, was done to amplify inserts, which were then used as probes by marking with ^{32}P[dATP] to hybridize them with genomic restrictions of the cassava parents described above.

Results

The 10 RGAs were successfully multiplied in the DH5-α *E. coli* strain, using Cell-Porator® Voltage Booster from Gibco-BRL at 2.4 kV/cm^2. The transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media. The complete digestion of genomic DNA was observed, using the six enzymes indicated above.

Southern blot analysis for each enzyme and genotype was performed. All filters were then hybridized with seven different probes from rice and maize, labeled with ^{32}P. The probe Pic 15, a NBS gene from maize, showed bands with very low hybridization to both parents, at different molecular weight with *Eco*RV (1500 bp for CM 2177-2 and 1600 bp for M Nga 2), *Hind*III (1600 bp for CM 2177-2 and 1500 bp for M Nga 2), *Dra*I (1400 bp for CM 2177-2 and 1500 bp for M Nga 2) (Figure 7.7). The process was repeated, but no hybridization was achieved, probably because of technique sensibility (Figure 7.8 B). Hibridization of a cassava probe used as control, to cassava genome restricted with the mentioned enzymes, is showed in Figure 7.8.A. In conclusion, these monocotyledonous probes have too low homology with cassava DNA. We are therefore continuing with degenerated primers based on disease resistance genes from crops other than cassava.

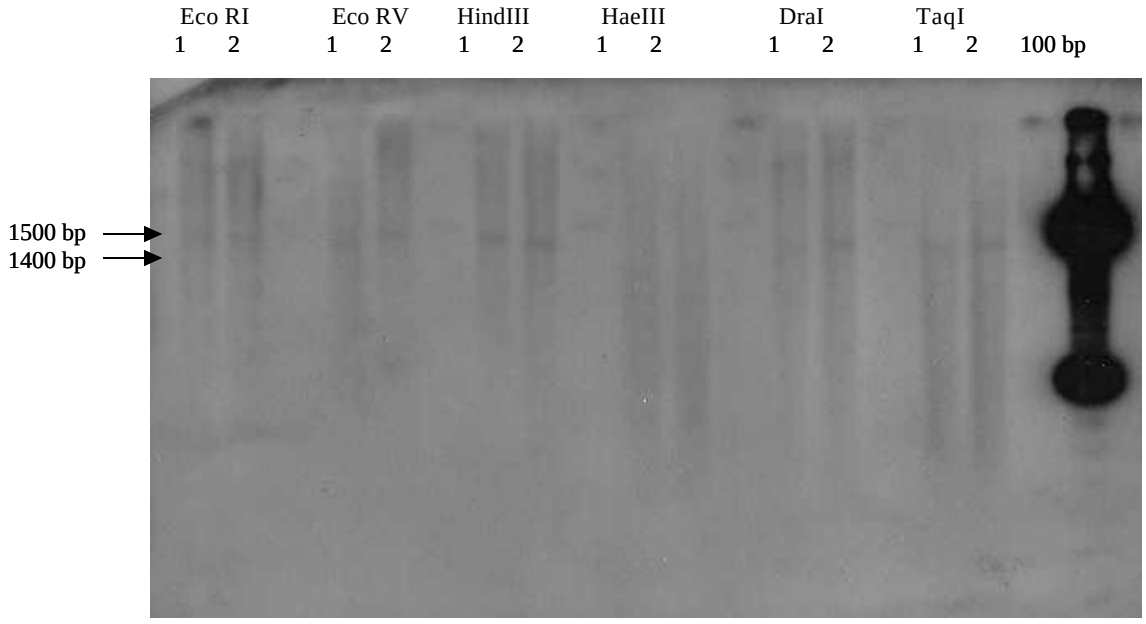

Figure 7.7. Hybridization of probe Pic 15 from maize to DNA digested with six enzymes from CM 2177-2 (1) and M Nga 2 (2), parents of the K family of cassava.

(A) (B)

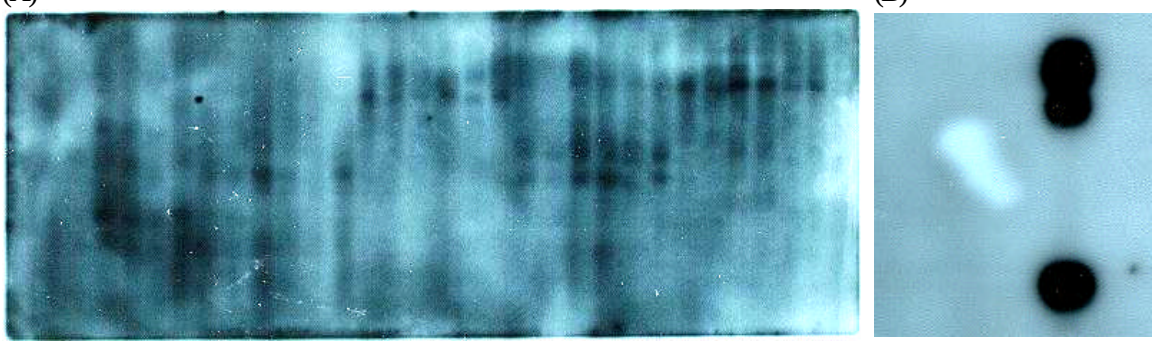

Figure 7.8. **(A)** Hybridization of a control probe from the cassava genetic map to different cassava genotypes. **(B)** Hybridization of the monocotyledonous probe Pic 21 to a 100-bp ladder on a membrane where family K parents DNA was transferred.

Reference

Dellaporta SL; Wood J; Hicks JR. 1983. A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19.

Activity 7.9. Using PCR with degenerated primers to search for resistance gene analogs associated with resistance to cassava bacterial blight

Specific objective

1. To develop molecular markers associated with resistance to CBB.

Methodology

A set of five primers used in rice by Chen et al. (1998) and corresponding to conserved domains in disease resistance genes were used to amplify similar sequences in cassava DNA from CBB-resistant genotypes. Each PCR reaction was performed in 25-µL volumes, consisting of dATP, dCTP, dGTP, and dTTP at 0.2 mM each; 2.5 mM $MgCl_2$; 0.25X Q solution (QIAGEN kit for PCRs); 1.5 U of *Taq* polymerase; 1 µM primer; 2.5 µL 10X *Taq* polymerase buffer; and 150 ng template DNA. For control reactions, template DNA was substituted by sterilized distilled H_2O.

Amplification of NBS, Pto, WipK, and XLLR was carried out in a MSJ-Research PTC-100 thermal cycler programmed for 5 min at 94°C; 45 cycles with denaturing for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 2 min at 72°C; and a final extension for 7 min at 72°C. For amplification with the KSU primer, the same program was used, but changing the annealing temperature to 42°C and the final extension time to 10 min.

The primer NBS is a sequence from conserved motifs of the nucleotide-binding site in tobacco N and *Arabidopsis* RPS2 gene (Yu et al. 1996); XLRR is a sequence based on the leucine-rich

repeat region of the RPS2 and Xa 21 from rice (Chen et al. 1998); Pto is a sequence for potato kinase (Leister et al. 1996); WipK amplifies the conserved region of MAK kinase from parsley (Y12875), tobacco (D61377), *Arabidopsis* (MPK3), and *Medicago sativa* (MMK4) (Ligterink et al. 1997); and KSU is a sequence recommended by Dr Hulbert Scot, Kansas State University.

Primers used were:

XLRR f: 5'-CCGTTGGACAGGAAGGAG-3'
XLRR r: 5'-CCCATAGACCGGACTGTT-3'

WipK 1: 5'-GGTCGTGGTGCTTATGGAAT-3'
WipK 2: 5'-CCATGAAGATGCAACCGAC-3'

NBS f1: 5'-GGAATGGGNGGNGTNGGNAARAC-3'
NBS r1: 5'-YCTAGTTGTRAYDATDAYYYTRC-3'

Pto 1: 5'-ATGGGAAGCAAGTATTCAAGGC-3'
Pto 2: 5'-TTGGCACAAAATTCTCATCAAGC-3'

KSU f: 5'-GGIGGIGTIGGIAAIACIAC-3'
KSU r: 5'-ARIGCTARIGGIARICC-3'

DNA (150 ng) from three cassava genotypes resistant to CBB (CM 6438-14, CM 7772-13, and CM 3311-4) and from one susceptible genotype (M Bra 1045) was amplified with the primers described. The PCR product was electrophoresed in 1.8% agarose gel in 0.5X TBE buffer. A 100-bp DNA ladder was used to estimate the size of each amplified DNA fragment.

The PCR product was purified, using QIAquick PCR purification kit (QIAGEN). To search for sequences associated with NBS genes, well-defined bands between 350 and 800 bp, obtained by PCR with the degenerated NBS primer, were eluted from agarose gel, using QIAquick Gel extraction kit (QIAGEN).

PCR products and eluted bands were introduced into the DH5-α *E. coli* strain, by electroporation at 2.4 kV/cm^2. Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media and conserved in glycerol at -80°C.

Different sized bands were observed by restriction with enzyme *Eco*RI from the vector and electrophoresed in 1.5% agarose gel in 0.5X TBE buffer.

Some clones will later be sequenced to search for homologies with disease resistance genes reported in GenBank (www.ncbi.nlm.nih.gov) and, using sequence-matching resistance genes, primers will be designed to amplify DNA from a segregant population, using SSCP analysis.

Results

Cloning. PCR products from three cassava genotypes resistant to CBB (CM 6438-14, CM 7772-13, and CM 3311-4) and from one susceptible genotype (M Bra 1045) were amplified with the primers described above (Figure 7.9). The PCR product was electrophoresed in 1.8%

agarose gel in 0.5X TBE buffer. A 100-bp DNA ladder was used to estimate the size of each amplified DNA fragment.

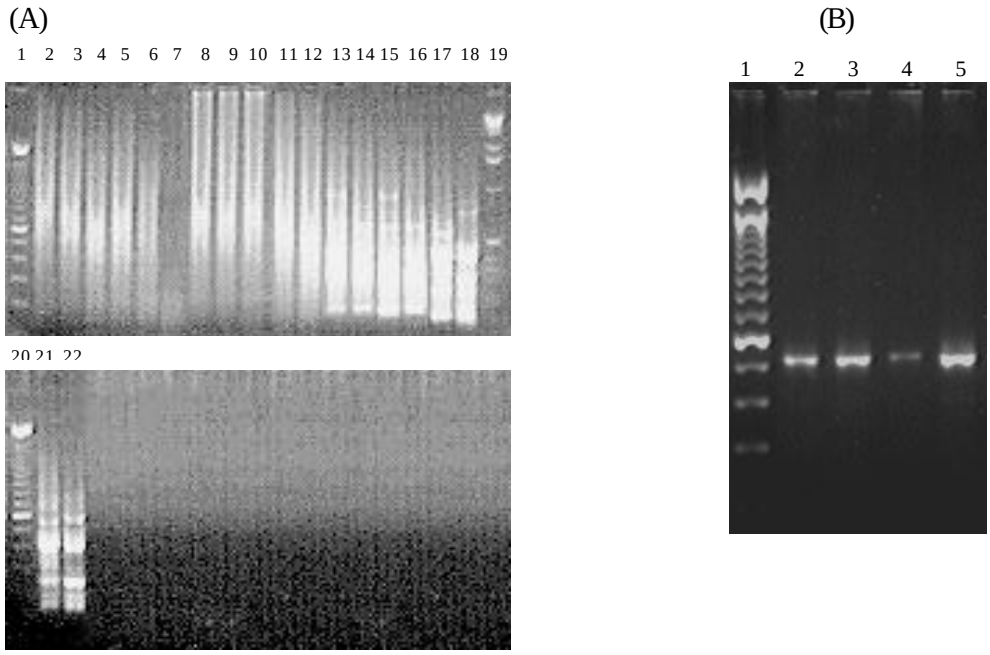

Figure 7.9. DNA from CM 6438-14, CM 7772-13, CM 3311-4 and M Bra 1045 amplified with primers NBS, Pto, WipK, and XLLR. **(A)** *Primer NBS:* lane 1 = 100 bp; lane 2 = CM 6438-14; lane 3 = CM 7772-13; lane 4 = CM 3311-4; lane 5 = M Bra 1045; lane 6 = positive control (M Cr 81); lane 7 = negative control. *Primer Pto:* lane 8 = CM 6438-14; lane 9 = CM 7772-13; lane 10 = CM 3311-4; lane 11 = M Bra 1045; lane 12 = positive control (M Cr 81). *Primer WipK:* lane 13 = CM 6438-14; lane 14 = CM 7772-13; lane 15 = CM 3311-4; lane 16 = M Bra 1045. *Primer XLLR:* lane 17 = CM 6438-14; lane 18 = CM 7772-13; lane 19 = 1 kb; lane 20 = 100 bp; lane 21 = CM 3311-4; lane 22 = M Bra 1045. **(B)** *Primer KSU*: lane 1 = 100 bp; lane 2 = CM 7772-13; lane 3 = CM 3311-4; lane 4 = CM 6438-14; lane 5 = M Bra 1045**.**

Band elution and cloning. Well-defined bands between 350 and 800 bp were obtained by PCR with a degenerated NBS primer. These were then eluted from agarose gel (Figure 7.5, inside circle, lane 2).

The number of clones obtained with the primers 6 (WipK), 13 (XLLR), 7 (Pto), and 1 (NBS) from DNA amplification of resistant genotypes CM 6438-14, CM 7772-13, and CM 3311-4 (Table 7.13).

Table 7.13. Clones obtained by PCR from CM 6438-14, CM 7772-13, and CM 3311-4 with primers NBS, Pto, WipK, and XLRR.

Clone	Primer	Size (bp)	Genotype	Clone	Primer	Size (bp)	Genotype
N36	NBS	600	CM 7772-13	X1	XLRR	530	CM 3311-4
P30	Pto	350	CM 3311-4	X2	XLRR	700	CM 3311-4
P31	Pto	390	CM 3311-4	X3	XLRR	750	CM 7772-13
P32	Pto	420	CM 3311-4	X4	XLRR	310	CM 7772-13
P33	Pto	330	CM 3311-4	X5	XLRR	1150	CM 7772-13
P34	Pto	380	CM 3311-4	X6	XLRR	390	CM 7772-13
P35	Pto	580	CM 3311-4	X7	XLRR	370	CM 6438-14
P36	Pto	530	CM 3311-4	X8	XLRR	500	CM 6438-14
W1	WipK	1200	CM 3311-4	X9	XLRR	400	CM 6438-14
W2	WipK	480	CM 3311-4	X10	XLRR	450	CM 6438-14
W3	WipK	420	CM 3311-4	X11	XLRR	280	CM 6438-14
W4	WipK	230	CM 3311-4	X12	XLRR	600	CM 6438-14
W5	WipK	610	CM 7772-13	X13	XLRR	580	CM 6438-14
W6	WipK	300	CM 6438-14				

Different sized bands were observed by restriction with enzyme *Eco*RI from the vector and electrophoresed in 1.5% agarose gel in 0.5X TBE buffer (Figure 7.11).

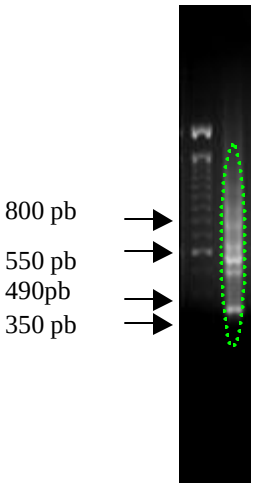

Figure 7.10. Bands obtained by PCR with a degenerated NBS primer and eluted from agarose gel (inside circle, lane 2). Lane 1 = 100 bp; lane 2 = CM 7772-13 amplified with NBS.

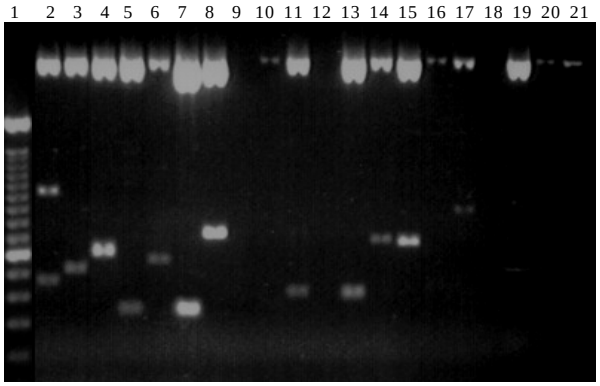

Figure 7.11. Different-sized bands observed by restriction with enzyme *Eco*RI from pGEM-T Easy vector. Lane 1 = 100 bp; lanes 2-8 = different CM 3311-4 clones; lane 9 = CM 7772-13 amplified with WipK; lanes 10-12 = different CM 6438-14 clones amplified with WipK; lanes 13-20 = different CM 3311-4 clones amplified with XLLR; lane 21 = CM 7772-13 amplified with XLLR.

Clones N-36, P-36, W-1, W-2, W-5, X-1, X-3, X-5, X-8, X-9, X=10 and X-12 were sequenced from the plasmid, using T7 and SP6 primers. No homologies were found in GenBank database to any disease resistance gene.

References

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Ligterink W; Kroj T; Zur Nieden U; Hirt, H.; Scheel D. 1997. Receptor-mediated activation of a MAP kinase in pathogen defense of plants. Science 176:2054-2057.

Yu YG; Buss GR; Saghai Maroof MA. 1996. Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. In: Proc Natl Acad Sci (USA) 93:11751-11756.

Activity 7.10. Using PCR with degenerated primers to search for resistance gene analogs associated with resistance to Phytophthora Root Rot

Specific objective

1. To develop molecular markers associated with resistance to PRR.

Methodology

Three sets of primers used in rice by Chen et al. (1998) (*see* Activity 7.8), corresponding to conserved domains in disease resistance genes, were used to amplify similar sequences in

cassava DNA from genotypes resistant to *Phytophthora* spp. As in Activity 7.8, each PCR reaction was performed in 25-µL volumes, consisting of dATP, dCTP, dGTP, and dTTP at 0.2 mM each; 2.5 mM $MgCl_2$; 0.25X Q solution (QIAGEN kit for PCRs); 1.5 U of *Taq* polymerase; 1 µM primer; 2.5 µL 10X *Taq* polymerase buffer; and 150 ng template DNA. For control reactions, template DNA was substituted by sterilized distilled H_2O.

Amplification was carried out in a MSJ-Research PTC-100 thermal cycler programmed for 5 min at 94°C; 45 cycles with denaturing for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 2 min at 72°C; and a final extension for 7 min at 72°C. For amplification with KSU primer, the same program was used, but changing the annealing temperature to 42°C and the final extension time to 10 min.

The primer NBS is a sequence from conserved motifs of the nucleotide-binding site in tobacco N and *Arabidopsis* RPS2 gene (Yu et al. 1996); Pto is a sequence for potato kinase (Leister et al. 1996); KSU is a sequence recommended by Dr Hulbert Scot, Kansas State University.

Primers used were:

NBS f1: 5'-GGAATGGGNGGNGTNGGNAARAC-3'
NBS r1: 5'-YCTAGTTGTRAYDATDAYYYTRC-3'

Pto 1: 5'-ATGGGAAGCAAGTATTCAAGGC-3'
Pto 2: 5'-TTGGCACAAAATTCTCATCAAGC-3'

KSU f: 5'-GGIGGIGTIGGIAAIACIAC-3'
KSU r: 5'-ARIGCTARIGGIARICC-3'

DNA from three cassava varieties resistant to *Phytophthora* spp. (M Bra 1045, M CR 81, and M Bra 532) was amplified with the primers described. The PCR product was electrophoresed in 1.8% agarose gel in 0.5X TBE buffer. A 100-bp DNA ladder was used to estimate the size of each amplified DNA fragment.

To search for sequences associated with NBS genes, well-defined bands between 350 and 800 bp, obtained by PCR with the degenerated NBS primer, were eluted, using a QIAGEN kit. PCR products and eluted bands were ligated in a pGEM-T Easy vector, which was introduced into the DH5-α *E. coli* strain by electroporation at 2.4 kV/cm^2. Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media.

Positive inserts were observed by plasmid restriction with *Eco*RI and electrophoresis in 1.5% agarose gel. Different sized fragments were selected to sequence by automated dideoxy sequencing (ABI Prism 377-96 DNA sequencer) and analyzed with Sequencher 4.1 software. Sequences were matched by nucleotide-protein sequence homology, using Blastx, a tool in GenBank (www.ncbi.nlm.nih.gov). Primers were designed, using Primer 3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

Results

Cloning. PCR products (Figure 7.12) were ligated in PGEM-T Easy vector. Transformant strains of *E. coli* DH 5-α were obtained by electroporation and conserved in glycerol at -80 ºC. Different size bands were observed by restriction with enzyme *Eco* RI from the vector and electrophorized in 1.5% agarose gel (Figures 7.13. and Table 7.14). PCR was done in some recombinant plasmids with double fragment observed when restricted by *Eco* RI. A total of 27 clones were obtained with NBS primer, two clones with Pto primer by DNA amplification of resistant genotypes M Bra 1045 and M Cr 81 and one with KSU primer by DNA amplification of resistant genotype M Bra 532.

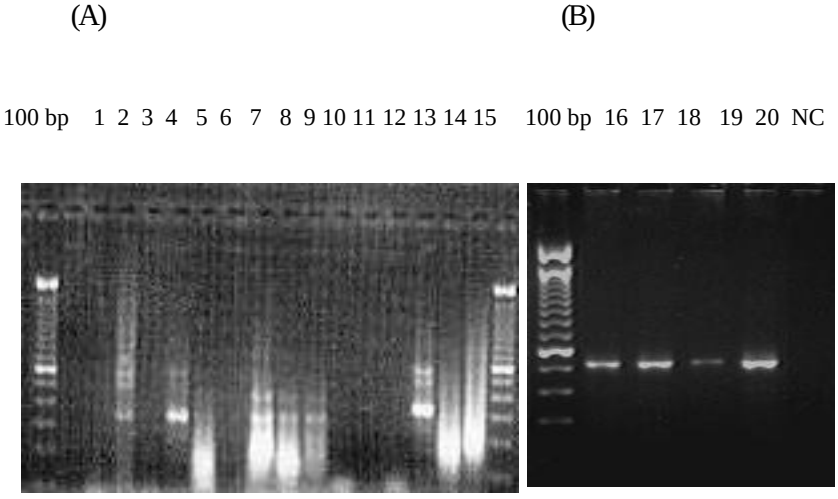

Figure 7.12. DNA from cassava genotypes (M Bra 1045 and M CR 81) resistant to Phytophthora root rot and amplified with primers NBS, Pto, and KSU. **(A)** *Primer NBS*: lane 1 = M Bra 1045; lane 2 = M CR 81; lane 3 = M Bra 1045; lane 4 = M CR 81; lane 6 = negative control; lane 9 = M Bra 1045; lane 10 = M CR 81; lane 11 = M Bra 1045; lane 12 = M CR 81. *Primer Pto*: lane 5 = M Bra 1045; lane 7 = M CR 81; lane 8 = M CR 81; lane 13 = M Bra 1045; lane 14 = M CR 81; lane 15 = M CR 81. **(B)** *Primer KSU:* lane 16 = CM 7772-13; lane 17 = CM 3311-4; lane 18 = CM 6438-14; lane 19 = M Bra 1045; lane 20 = M CR 81; NC = negative control.

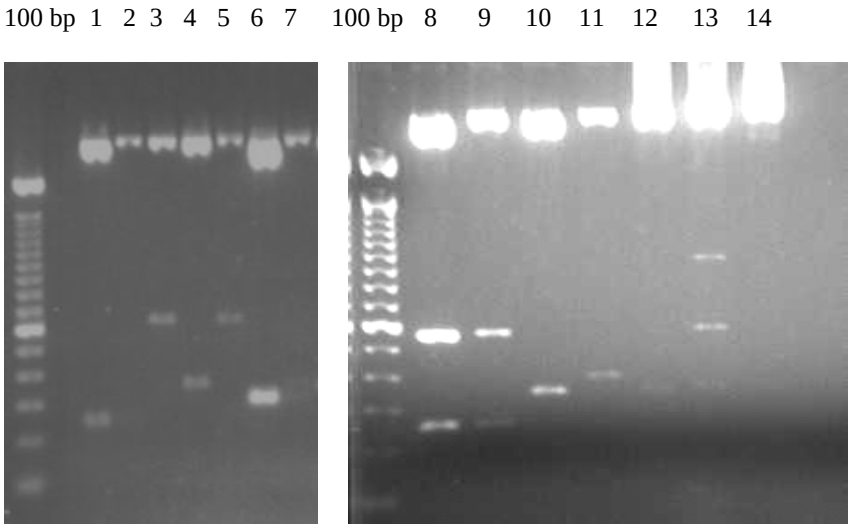

Figure 7.13. Inserts obtained from M CR 81 and M Bra 1045 by PCR with a degenerated NBS primer, cloned in pGEM-T Easy vector and excised with *Eco*RI. Lane 1 = clone 1-2; lane 2 = clone 1-3; lane 3 = clone N-32; lane 4 = clone N-33; lane 5 = clone 2-3; lane 6 = clone N-34; lane 7 = clone 4-1; lane 8 = clone 1-4; lane 9 = clone 1-5; lane 10 = clone 2-4; lane 11 = clone 2-5; lane 12 = clone 2-6; lane 13 = clone 4-3; lane 14 = clone 4-4.

Table 7.14. Clones obtained by PCR from cassava varieties M CR 81 and M Bra 1045 with NBS and Pto primers.

Clone	Genotype	Primer	Size (bp)	Clone	Genotype	Primer	Size (bp)
N-13	M Bra 1045	NBS	131	N-35	M Cr 81	NBS	1100
N-15	M Bra 1045	NBS	300	N-37	M Bra 1045	NBS	325
N-16	M Bra 1045	NBS	866	N-38	M Bra 532	NBS	474
N-17	M Bra 1045	NBS	280	1-2	M Cr 81	NBS	270
N-18	M Bra 1045	NBS	873	1-4	M Cr 81	NBS	800
N-20	M Bra 1045	NBS	120	1-5	M Cr 81	NBS	800
N-21	M Bra 1045	NBS	120	1-5-1	M Cr 81	NBS	800
N-22	M Bra 1045	NBS	500	1-5-2	M Cr 81	NBS	800
N-23	M Bra 1045	NBS	464	2-3	M Bra 1045	NBS	650
N-24	M Bra 1045	NBS	893	2-5	M Bra 1045	NBS	400
N-30	M Cr 81	NBS	950	4-1	M Cr 81	NBS	300
N-31	M Cr 81	NBS	950	4-3	M Cr 81	NBS	1600
N-32	M Bra 1045	NBS	650	P-10	M Bra 1045	Pto	400
N-33	M Bra 1045	NBS	502	P-24	M Bra 1045	Pto	440
N-34	M Cr 81	NBS	320	K-1	M Bra 532	KSU	496

Band elution and cloning. Figure 7.14 shows eluted bands, which were obtained by PCR of M CR 81 DNA with a degenerated NBS primer. No positive clones were obtained after cloning in *E. coli*.

Sequencing. Some clones were sequenced to search for homologies with disease-resistance genes reported in GenBank (www.ncbi.nlm.nih.gov). With sequence-matching resistance genes, primers were designed to amplify DNA from a segregant population. The sequence obtained from clones N-23, N-33, N-37, N-38 and K-1 with T7 primer are reported in Figure 7.15, and their matching scores are described in Table 7.15, where homology with diverse disease resistance gene products is presented. Table 7.15 thus shows a list of main resistance gene analogs to sequenced clones N-23, N-33, N-37, N-38 and K-1, which were matched by nucleotide-protein sequence homology, using Blastx, a tool in GenBank. Sequences obtained matches with NBS, NBS/LRR, NBS/Toll and disease resistance genes reported for different species.

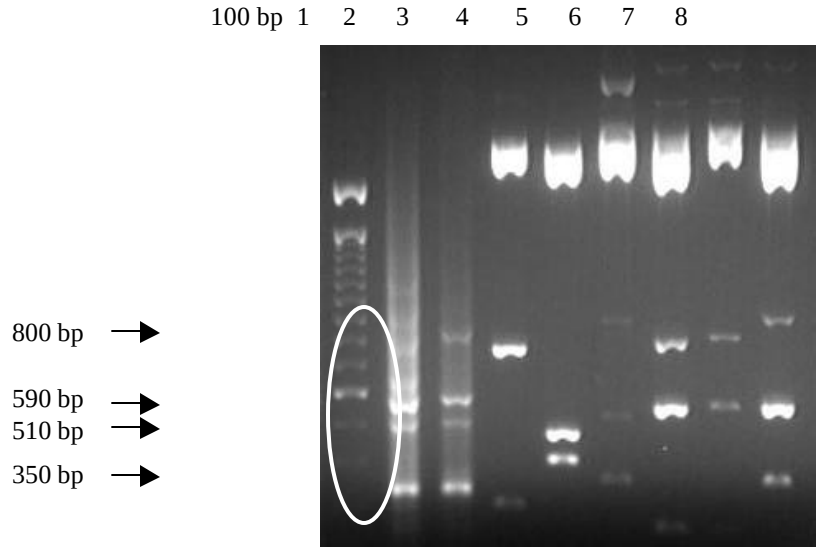

Figure 7.14. Bands obtained by amplification of DNA from M CR 81 with a degenerated NBS primer and eluted from agarose gel (inside circle, lane 2). Clones in the pGEM-T Easy vector were restricted with *Eco*RI (lanes 3-8).

(A)

1 GAATGGGGGT CGTCGGGAGAACAACTATTGC TAGACKDGGMTRTSAGCMACTATCCYCTC
61 AATWWGAAGGTAGCAGCTTTCTTKCAAATGTTAGAGAAGKTGGGGAGAAGTATGGTTWGG
121 WYYCTTWACAAAAACAGMTGCTTACTGCAATTTTAATTGATCCGKACATATCTATTTGCG
181 ATGCTCATAGTGSAKCTGATGAGGWCASAAKTGGGCTACMTGGGAAAAAAGKYCTARTMR
241 TKCTGGATGATGTCTGCCRATTGGACCAGTTAAAMTTWTTMRCTGGCATGCATGATWCGG
301 ATMSGGAATGGAAGCAAGGTAATCMTSACRACTNNAAATCMCTAGYKAAYTCACGGYCGY
361 CTGCANGTSCTCCATRTSSGAKAKCTCCCAACGCGCCKRTGCATMCCTTGAGTTTMTATA
421 TNGCCCCTACATAGCTTGACGTARTCATGGTCATAGGCGCTCCT

(B)

1 GGAATGGGAGGGGTGGGAAAGACAACTTTAGCTCAGCTTCTYTTTAATGAAGCCAAACTG
61 AACTTTGATTTAACGGCTTGGGTTTTAKTTGGKGATGATTTTGACGTTTTCARAATCTCC
121 CAAACGATTTTCCAGTGGTTTGGGGGRRATTTTGATGGCAAARATTTGAATTTGCTTYAA
181 GTAAGATTGAAGGAAAAGCTKTCCCATAARAAATTTTTGATTGKCCYGGATGACCTTTGG
241 AATGARAAGTWTGAGGRTTGGAATCYYTTTYGNGGGCCYTTTGAAWWTGGGGCAAGAGGA
301 ASCMGGGTAATCNTTCCMMCTAGRAATCMCTNTTTGAATTNNCGGCCNCCTGCAGGTCNA
361 CCATNTGGGANAGCNCCCMMCCCSGTTGGATGCNTANCTNGAGTNTTYTATATGGNCCCY
421 CAAWAGCTTKGCSTAATCATGGGCATAGCTGTTTCYGTGTGAAAATGKTATTCCCTCCCA
481 ATTYCMCMCAACAWNCCAGCCC

(C)

1 GGAATGGGGGGCGTAGGGAAGACAACTCTAGCTCAACTAGTCTACAATGATCCCATGTTG
61 GAGTTTGATTTAAAAGCCTGGGTGTCTGTTGGTGAAGATTTTGATGTTTCCAGGGTCACA
121 AAAACATTTCTTCTTCAACTGGGTGATGGCGGT0GATGATAAAGATTTGAATCTGCTTCAG
181 GTAAAATTGAAGCAAAAGTTGTCTGGGAAGAAGTTTTTAGTTGTCCTAGATGATGTCTGG
241 ACCCAGAACTATGAAGAATGGGCTCTATTTTGGGGTCCTTTTGAAGCAGGGGCTCCTCAA
301 AGCAAGATCATCATCACAACTAGAA

(D)

1 AGGGCTAGGGGGAGGCCAGGGCTAGGGCTAGGGCTAGGGGGAGGCCATTGGCGTAATCTA
61 CCACCTTACATGACAACCCCATATATTCTTTTGAAGGATGGTGATTTTGGAAGGCCCTTA
121 GACTTAAAAGTTGAAGGCCTTCAATATCATCTAGTCCTTTAGCCTTGGATATTTGATCCA
181 CTCCATGACCATTAAGCAAATGCTCATCTCTAGTA GTGATAAGAATTCTACTCCCTGGGC
241 CAAACCAATCATTTTTTCCGGCTAGTTTTTGCAACTGATTTAGTTCATTCACATCATCCA
301 TAACAATCAAGACCCTTTTGAGGCGAAGCTTCCTTTTTATCATGTCCATTCCATTATATA
361 TATTCCATATTTTAACCCTTTTCTCCGTAAGAATTTCTGAAAGAAGTTGTTCTTGTAAAG
421 AGACTAGGCCACCTTTAGATGAAACTTTCAMTAAACACTGGGAAAGGAAGGCYC

(E)

1 GGGGGGGTGGGGAAGACGACTTTTATACCAAGGATATATGCGGCCTCCCCCTAGCCCTAG
61 CCTGGGGGGGTGGGGAAGACSA CCATTGCAAGAGCTTAATACAATTCCGTATCTTATCAT
121 CAGTTTGAGGGTAAGGCCTTCCTTTCCAGTGTTAGAGAAGTTTCATCTAAAGGTGGCCTA
181 GTCTCTTTACAAGAACAACTTCTTTCAGAAATTCTTACGGAGAAAAGGGTTAAAATATGG
241 AATATATATAATGGAATGGACATGATAAAAAGGAAGCTTCGCTTCAAAAGGGTCTTGATT
301 GTTATGGATGATGTGAATGAACTAAATCAGTTGCAAAAACTAGCCGGAAAAAATGATTGG
361 TTTGGCCCAGGGAGTAGAATTCTTATCACTACTAGAGATGAGCATTTGCTTAATGGTCAT
421 GGAGTGGATCAAATATACAAGGCTAAAGGACTAGATGATATTGAAGGCCTTCAACTTTTA
481 AGTCTAAGGGCCTTYC

Figure 7.15. Sequences of cassava clones isolated from M Bra 1045 and M Bra 532 by PCR with degenerated NBS primers; **(A)** clone N-23 (464 bp); **(B)** clone N-33 (502 bp); **(C)** clone N-37 (325 bp); **(D)** clone N-38 (474 bp), isolated from MBRA 532 with a NBS primer; **(E)** clone K-1 (496 bp), isolated from MBRA 532 with KSU primer. D: A, G or T, K: G or T, M: A or C, N: any base, R: A or G, S: G or C, W: A or T, Y: C or T. Underlined bases show where each primer starts amplification.

Table 7.15. Main resistance gene analogs matching with clones 2-4, N-23, and N-33 isolated by PCR with a degenerated NBS primer from cassava genotype M Bra 1045.

Protein matching in GenBank	Matching species	Homology score (bits)	Prob. of higher scores	Identities (%)[a]	Positives (%)[b]
Clone N-23 (primer T7)					
NBS-kinase protein Z2	*Solanum tuberosum*	56.2	1e -07	35	47
Putative disease resistance gene analog NBS-LRR	*Malus prunifolia*	54.3	5e –07	40	47
Disease resistance-like protein	*Glycine max*	52.8	1e -06	35	45
Putative resistance gene homolog	*Cucumis melo*	51.6	3e –06	32	45
NBS-2	*Cucumis melo*	51.2	4e -06	32	45
Resistance-gene protein	*Vigna unguiculata*	50.4	7e –06	34	44
Resistance-like protein KNBS2	*Glycine max*	48.9	2e -05	35	43
Resistance-like protein KNBS3	*Glycine max*	48.9	2e -05	34	45
Putative resistance protein	*Glycine max*	48.9	2e -05	31	41
Resistance-gene protein	*Vigna unguiculata*	48.9	2e -05	33	44
Clone N-33 (primer T7)					
NBS/LRR resistance protein-like protein	*Theobroma cacao*	103	1e -21	44	59
Resistance protein candidate	*Lactuca sativa*	96.7	1e -19	46	60
Probable resistance protein-soybean (fragment)	*Glycine max*	93.2	1e -18	43	59
Disease resistance-like protein	*Glycine max*	92.4	2e -18	45	60
NBS-LRR resistance-like protein J78	*Phaseolus vulgaris*	92.0	2e -18	41	57
Disease resistance protein I2	*Lycopersicon esculentum*	88.2	4e -17	42	55
Putative resistance protein KNBS4	*Glycine max*	87.8	5e -17	41	58
Resistance gene analog	*S. phureja* × *S. stenotomum*	84.3	5e -16	40	57
Clone N-37 (primer T7)					
NBS/LRR resistance protein-like protein	*Theobroma cacao*	92.4	1e -18	44	53
Disease resistance protein homolog	*Vigna unguiculata*	86.7	5e -17	44	53
RGA -B protein	*Cicer arietinum*	82.8	8e -16	43	51
NBS-LRR resistance-like protein J78	*Phaseolus vulgaris*	80.5	4e -15	41	49
Clone N-38 (primer T7)					
Putative disease resistance-like protein NBS-LRR	*Malus domestica*	126	8e -29	46	70
Putative disease resistance protein OB8	*Phaseolus vulgaris*	125	2e -28	45	66
Putative disease resistance gene analog NBS-LRR	*Malus prunifolia*	125	2e -28	45	68
Putative NBS-LRR type disease resistance protein	*Pisum sativum*	125	2e -28	47	70

Putative resistance gene homologue	*Cucumis melo*	124	4e -28	43	64
R-gene homolog, similar to St334	*Solanum tuberosum*	120	4e -27	46	71
NBS-LRR-Toll resistance gene analog protein	*Medicago sativa*	110	8e -24	42	67
Clone K-1 (primer T7)					
Putative disease resistance gene analog NBS-LRR	*Malus prunifolia*	146	1e -34	52	72
Resistance-like protein KNBS3	*Glycine max*	142	2e -33	50	74
Resistance-gene protein	*Vigna unguiculata*	135	2e -31	52	72
Putative disease resistance-like protein NBS-LRR	*Malus domestica*	132	1e –30	48	73
Resistance protein analog	*Phaseolus vulgaris*	133	9e –31	49	66
NBS-LRR-Toll resistance gene analog protein	*Medicago sativa*	132	2e –30	50	72

a. Identities = matching gene products.
b. Positives = matching nucleotide.

Primer design. Based on the sequences obtained, primers were designed, using Primer 3 software (wwwgenome.wi.mit.edu/cgibin/primer/primer3_www.cgi). Primer characteristics are presented in Table 7.16. In Figure 7.15, underlined bases show where each primer starts amplification.

Table 7.16. Primers designed to amplify disease-resistant sequences from cassava K family.

Primer	Base starting	Length bp	Melting temp. °C	GC %	Product size (bp)
From clone N-23					
CGTCGGGAGAACAACTATTGC	11	21	61.91	52.4	453
GGAGCGCCTATGACCATGA	463	19	62.15	57.9	
From clone N-33					
TGATTTAACGGCTTGGGTTT	66	20	59.45	40.0	390
GAAACAGCTATGCCCATGATT	455	21	59.06	42.9	
From clone N-37					
GGGAAGACAACTCTAGCTCAACT	16	23	58.24	47.8	325
GATGATGATCTTGCTTTGAGGA	315	22	59.27	40.9	
From clone N-38					
GCCATTGGCGTAATCTACCA	44	20	60.86	50.0	400
TTCATCTAAAGGTGGCCTAGTCTC	443	24	60.15	45.8	
From clone K-1					
CCATTGCAAGAGCTTAATACAATTC	83	25	60.37	36.0	351
TTTGATCCACTCCATGACCA	433	20	59.89	45.0	

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Leister D; Ballvora A; Salamini F; Gebhardt CA. 1996. PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genet 14:421-429.

Yu YG; Buss GR; Saghai Maroof MA. 1996. Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. In: Proc Natl Acad Sci (USA) 93:11751-11756.

Activity 7.11. Evaluating cassava genotypes and families for resistance to Phytophthora root rot

Specific objectives

1. *To evaluate Phytophthora sp. resistance of a group of cassava landraces from Mitú.*
2. *To evaluate Phytophthora sp. resistance of some elite cassava cultivars.*
3. *To evaluate Phytophthora sp. resistance of two cassava families.*

Methodology: Mitú landraces

Roots from 24 cassava landraces collected from indigenous settlements at Mitú, Vaupés, and 3 genotypes adopted by indigenous farmers through participatory research were inoculated with fungal discs of the *Phytophthora* sp. isolate MTR4, also collected in Mitú. Root damage was determined by measuring width and length of lesions 7 days after inoculation. Rotten area of roots was calculated.

Methodology: elite genotypes

Ten elite genotypes and some commercial and local varieties from the Department of Quindío and Santander de Quilichao (Department of Cauca) were inoculated with fungal discs of *Phytophthora tropicalis*. Root damage was determined 5 days after inoculation by measuring width and length of lesions in transverse cuttings by the inoculation hole.

Methodology: cassava families CM 9582 and CM 9600

Cassava families CM 9582 (M Bra 1045 × M CR 81) and CM 9600 (M CR 81 × M CR 54), comprising 28 and 17 genotypes respectively, were characterized by root rot resistance after inoculation of fresh roots with fungal discs of *Phytophthora tropicalis*. Root damage was determined by measuring width and length of lesions 5 days after inoculation. A frequency analysis and graph with resistance distribution were completed.

Table 7.17. Twenty-eight cassava genotypes from Mitú, Vaupés, characterized for their resistance to *Phytophthora* sp., isolate MTR4.

Variety	Rotten area (cm^2)	Root rot (%)	Variety	Rotten area (cm^2)	Root rot (%)
Abeja	1.04	8.21	Siringa	4.28	18.02
Abiyú	2.32	11.04	Totuma	0.78	3.50
Dulce Cucura	2.07	10.33	Tres Mesina Dulce	4.06	17.02
Flores	1.17	7.35	Wasoco	0.25	1.41
Guaracú	2.00	8.92	Yuca de Agua	2.27	8.99
Hoja de Plátano	0.71	6.84	Yuca de Garza	3.18	18.22
Ibacaba	1.96	12.19	Yuca de Mico (white)	2.94	7.33
Inayá	1.49	10.06	Yuca de Mico (red)	3.65	14.43
Lapa Blanca	5.15	17.53	Yuca de Piña	3.18	15.05
Mirití	2.95	6.06	Yuca de Rana	2.92	14.62
Nupará	6.65	39.68	CG 165-7	3.39	27.30
Pintadillo	3.60	14.01	M Bra 12	5.65	18.89
Pupuña	3.23	8.83	M Bra 1044	3.08	9.04
Santa Catalina	0.70	5.00	M Ven 25	2.33	6.30
Duncan 5%	3.04	11.77	Duncan 5%	3.04	11.77

Results: Mitú landraces

Varieties Wasoco, Totuma, Santa Catalina, and Mirití were highly resistant to the pathogen, with the percentage of rot ranging from 1.4% to 6.05%, whereas 'Nupará', 'Yuca de Garza', and 'Siringa' were the most susceptible, having areas of rotten root similar to those of the susceptible control (M Bra 12), with percentages ranging from 18% to 39.7%. The control varieties—M Ven 25, M Bra 1044, and CG 165-7—adopted by indigenous farmers through participatory research, had rot percentages of 6.3%, 9.04%, and 27.3%, respectively (Table 7.17).

Results: elite genotypes

Of the 10 elite genotypes, M Bra 71, M Bra 1045, M Bra 532, CM 6438-14, and CM 3311-4 were the most resistant genotypes, with the percentage of rotten root area ranging from 7.17% to 15.3%. 'Manzana', a local variety from the Department of Quindío, had 28.11% of root area affected, as opposed to its performance in the field, where it was highly susceptible. 'Verde', a local variety from Santander de Quilichao (Department of Cauca), had 23.24% root area rotten. CM 7772-13, CM 523-7 (ICA Catumare), CM 2177-2 (ICA Cebucán), SM 1210-4, and M Col 2066 (Chiroza) were the most susceptible genotypes, with the percentage of rot ranging from 28% to 47.8% (Table 7.18). M Col 2066 in the field is susceptible to root rot.

Table 7.18. Characteristics of some commercial, elite, and local varieties of cassava genotypes for their resistance to *Phytophthora tropicalis* by artificial inoculation of roots in the laboratory.

Genotype	Rotten area (cm^2)	Root rot (%)	Genotype	Rotten area (cm^2)	Root rot (%)
Commercial genotypes			Genotypes for resistance studies		
CM 523-7	8.09	29.44	CM 7514-7	4.75	21.23
CM 2177-2	12.81	28.88	CM 7772-13	6.07	47.84
CM 3306-4	4.86	22.62	M Bra 12	5.81	26.59
HMC-1	4.86	22.77	M Bra 71	1.48	7.18
CM 3311-4	5.64	15.30	M Bra 532	1.25	10.29
			M Bra 1045	3.76	8.75
Elite Genotypes			M Nga 2	7.61	19.10
CM 2772-3	9.26	25.49			
CM 5655-4	8.13	17.07	Local varieties		
CM 6438-14	3.46	10.64	M Col 2066	10.71	28.10
M Bra 383	5.90	19.97	Manzana	3.44	24.82
M Per 183	7.34	19.84	Verde	8.75	23.24
SM 1210-4	5.45	28.11			
SM 1557-17	6.19	18.57			
SM 1741-1	5.22	16.03			
SM 653-14	3.67	16.33			
SM 909-25	6.81	26.60			
Duncan 5%	2.86	10.37	Duncan 5%	2.86	10.37

Results: cassava families CM 9582 and CM 9600

Disease severity was low, compared with the previous year's evaluation. Frequency analysis resulted in groupings according to percentage of root rotten area, as shown in the following list:

Group	CM 9582 family	CM 9600 family
1	0-6.8	0-10.5
2	6.8-13.5	10.5-21.0
3	13.5-20.3	21.0-31.5
4	20.3-27.0	31.6-42.0
5	27.1-33.8	42.1-52.5

As shown in Figure 7.16, 3.7% of individuals from the CM 9582 family are in groups 1 and 2, while 81.4% of individuals are in groups 3 and 4. Of the individuals in the CM 9600 family, 14.8% are in groups 1 and 2, while 25.9% are in group 5.

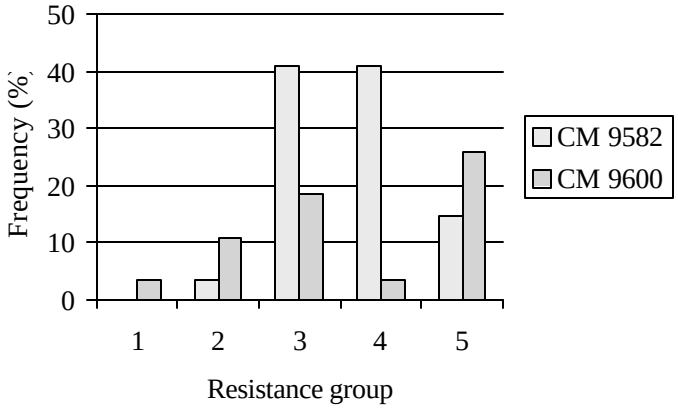

Figure 7.16. Breakdown of cassava families CM 9582 and CM 9600, inoculated with *Phytophthora tropicalis* on roots, according to their resistance groups, based on frequency analysis.

Activity 7.12. Genetics of Resistance to Rot Caused by Phytophthora tropicalis in Two Segregating Populations of Cassava (Manihot esculenta Crantz)

Rationale

In Brazil, of the *Phytophthora* spp., *P. drechsleri* Tucker most severely attacks cassava (Albuquerque and Figueiredo 1968). This species has been identified in Colombia (Oliveros et al. 1974), together with *P. nicotianae* var. *nicotianae* (Sánchez 1998; Lozano and Loke 1994;

Soto et al. 1988). Other species reported as cassava pathogens in different countries are *P. erythroseptica* (Fassi 1957), *P. cryptogea* (CIAT 1991), *P. meadii* and *P. arecae* (Alvarez et al. 1997; Barragán et al. 1998), and *P. tropicalis* (which is similar to *P. capsici*).

The development of *Phytophthora* spp. is favored by use of inadequate agronomic practices and ineffective fungicides, transport of material from affected areas to those free of the pathogen, and by planting in compact or very clayey soils (Takatsu and Fukuda 1990).

Currently, CIAT selects for resistance to *Phytophthora* spp. under greenhouse conditions, inoculating shoots and roots with isolates that were previously identified by sequencing the ITS region in the rDNA.

Molecular techniques are increasingly being used to decipher the genetic base of complex agronomic traits. Genetic improvement for disease resistance can be achieved more quickly and effectively by using molecular markers.

To better understand the genetics of resistance to *Phytophthora* spp., this study evaluates individuals from the cassava populations K family and CM 9582 for their reaction to root rot caused by *P. tropicalis*.

Materials and Methods

Plant materials

In 2000 and 2001, 1-year-old roots of 69 cassava genotypes belonging to the K family grown at CIAT's experiment station at Santander de Quilichao (Department of Cauca) were inoculated and evaluated. In July-August 2001, 1-year-old roots of 43 cassava genotypes belonging to the CM 9582 population were harvested at the Centro de Investigación de la Caña de Azúcar de Colombia (CENICAÑA, Florida, Department of Valle) and evaluated. Also included in the study were four genotypes from CIAT's Quilichao station: one resistant (M Bra 1045) and three susceptible (M Col 2066, CM 2177-2, and M Nga 2) to *Phytophthora tropicalis* (*Pt*):

Parent	Origin	Reaction to *Pt*
K family		
M Nga 2	Nigeria	Susceptible
CM 2177-2	Hybrid, CIAT	Susceptible
CM 9582		
M CR 81	Costa Rica	Susceptible
M Bra 1045	Brazil	Resistant

For the QTL analysis, roots of 92 genotypes of the K family were harvested at CIAT in 2000. The roots were then washed with drinking water and detergent, and disinfected, first with 1% hypochlorite, then with 30% ethanol, each for 10 min. The roots were then dried with sterilized paper towels. The material that was disinfected but not inoculated the same day was stored (for a maximum of 24 h) in a cold room at 4°C until inoculated.

The pathogen
As inoculum, isolate 71 was used. It was identified, through sequencing the ITS region of ribosomal DNA, as *P. tropicalis* (which is similar to *P. capsici*). This isolate was found in cassava infected with root rot in Barcelona (Department of Quindío). The inoculum was cultured in medium prepared with oat agar (2% Quaker® oats, 2% agar) and antibiotics (penicillin at 900 mg/mL; rifampicin, 0.2 g/mL; and ampicillin, 750 mg/mL). Incubation was carried out at temperatures between 20°C and 26°C for 4 to 11 days for the K family and 6 to 7 days for the CM 9582 population.

Inoculation
Within an isolation chamber, in front of a burner, a piece of cassava root, about 15 mm long, was extracted with a punch, 7 mm in diameter. At the bottom of the perforation left behind, a piece of the fungus, also extracted with a punch, 5 mm in diameter, was deposited. The extracted piece of cassava root was replaced and secured with masking tape. Each genotype was also inoculated with a negative control, that is, the medium of oat agar and three antibiotics, but no *P. tropicalis*. Once inoculated, the treated cassava roots were deposited in plastic bags, containing moist, sterilized, paper towels. The closed bags were then placed in plastic trays, and left at 22°C in darkness for 7 (K family) or 5 days (CM 9582).

Evaluation
From each cassava root, a cross section was taken at the point where the inoculum was deposited. The height and width of both wound and entire cross section were measured, together with root length, and depth of inoculum in the root. The type of rot was also evaluated: 1 = soft/moist; 2 = dry; 3 = soft/dry; 4 = soft/moist and dry. These data were recorded and processed through *Excel*'s calculation program.

Data analysis
The experimental unit was the root. For the K family, the following were taken into account: (1) genotypes with fewer than five (2000) or six (2001) roots were excluded; (2) roots with an average diameter of less than 3 cm were discarded; (3) slices of root with wounds wider than 7 (2001) or 8 cm (2000) were considered as having 100% of their area infected, and values for roots wider than 7 (2001) or 8 cm (2000) were converted for 7 or 8 cm, respectively.

For the CM 9582 population, the following were taken into account: (1) genotypes with fewer than four replicates were excluded; (2) roots with an average diameter of less than 3 cm were eliminated; (3) slices of root with wounds wider than 6 cm were considered as having 100% of their area infected, and values for roots wider than 6 cm were converted for 6 cm.

QTL analysis
One framework map was used for QTL analysis, based on the segregation of molecular markers in a population from a cross between two heterozygous parents: M Nga 2 (female) and CM 2177-2 (male). The female-derived map was based on the segregation of female alleles, corresponding to 192 markers that compromised RFLP, random amplified polymorphic DNA (RAPD), isoenzymes, microsatellites, expressed sequence tags (ESTs) and known genes (Fregene 2002, in preparation).

A significant association between a DNA marker and *Phytophthora* resistance was declared if the probability was more than 0.005 to minimize the detection of false positives. The degree of phenotypic variance explained by each marker was obtained from the regression coefficient (r^2 values). Total r^2 values from each QTL were computed as:

(sum of squares for each marker)/(total sum of squares)

All data were analyzed with Q-Gene on McIntosh.

Results

The roots of 69 individuals of the K family (M Nga 2 × CM 2177-2) and of 43 individuals of the CM 9582 population (M Bra 1045 × M CR 81) were inoculated with *P. tropicalis* to determine the genetic base of these populations' resistance to Phytophthora root rots (PRR).

The K family genotypes evaluated in 2000 and 2001 showed 30%-70% of areas continuously infected (Figure 7.17). Some genotypes that, in 2000, had intermediate resistance to *P. tropicalis* tended to become susceptible in 2001 and vice versa.

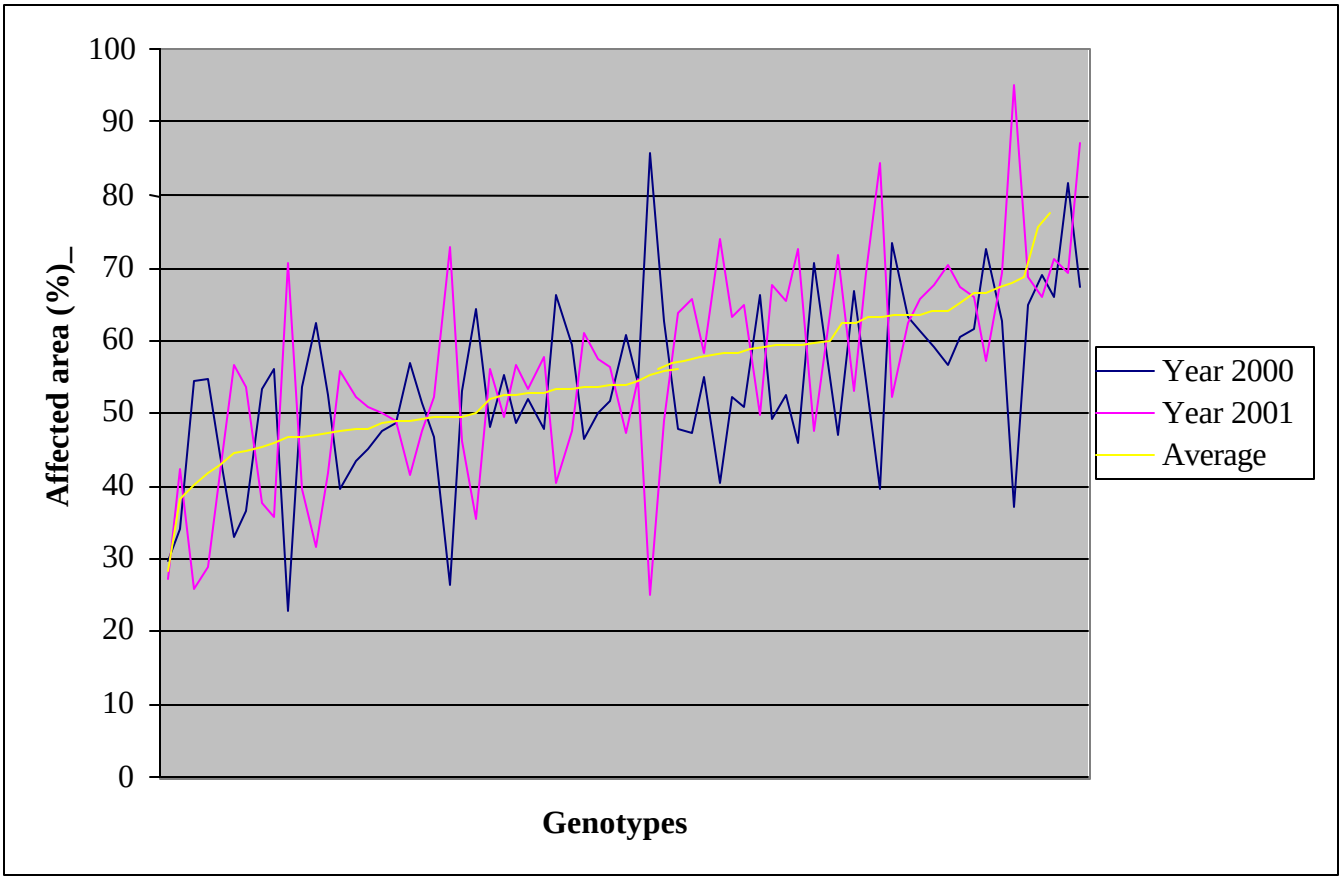

Figure 7.17. Percentage of area affected by the root rot fungus *Phytophthora tropicalis* across genotypes of the cassava K family for years 2000 and 2001.

Certain genotypes with intermediate resistance in 2000 continued presenting intermediate resistance in 2001, showing 30%-60% of area infected. Genotypes from the CM 9582 population (2001) maintained 70%-90% of areas continuously infected. Few genotypes had intermediate resistance (Figure 7.18). Figures 7.19 and 7.20 show the distribution of individuals by group, according to the degree of resistance to the pathogen. Resistant materials were not detected.

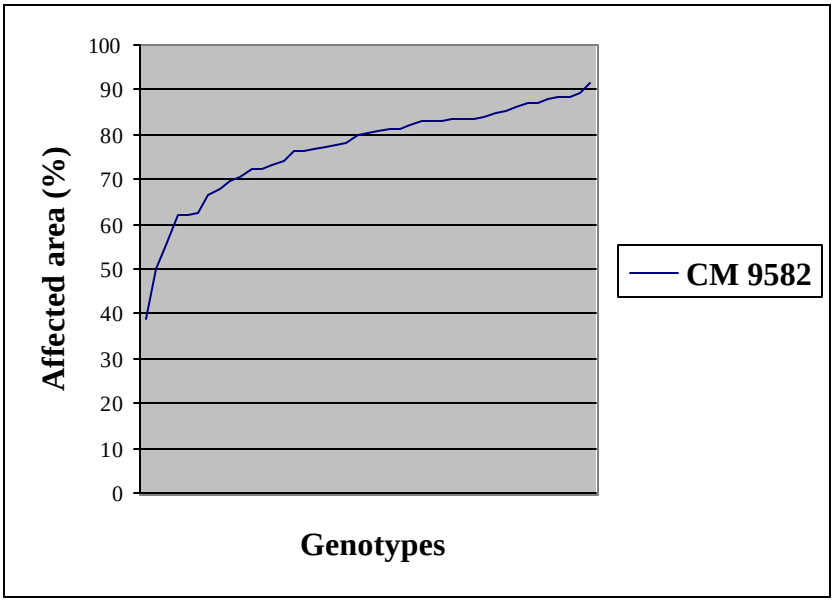

Figure 7.18. Percentage of area affected by the root rot fungus *Phytophthora tropicalis* across genotypes of the cassava CM 9582 population for year 2001.

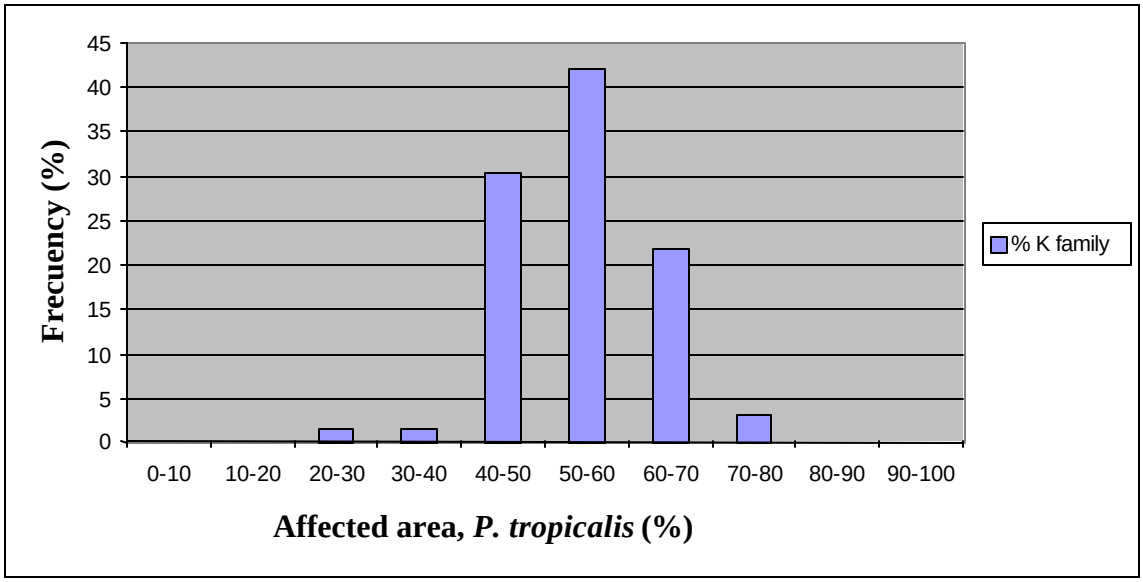

Figure 7.19. Distribution of the frequency of genotypes from the cassava K family according to the percentage of area affected by the root rot fungus *Phytophthora tropicalis*.

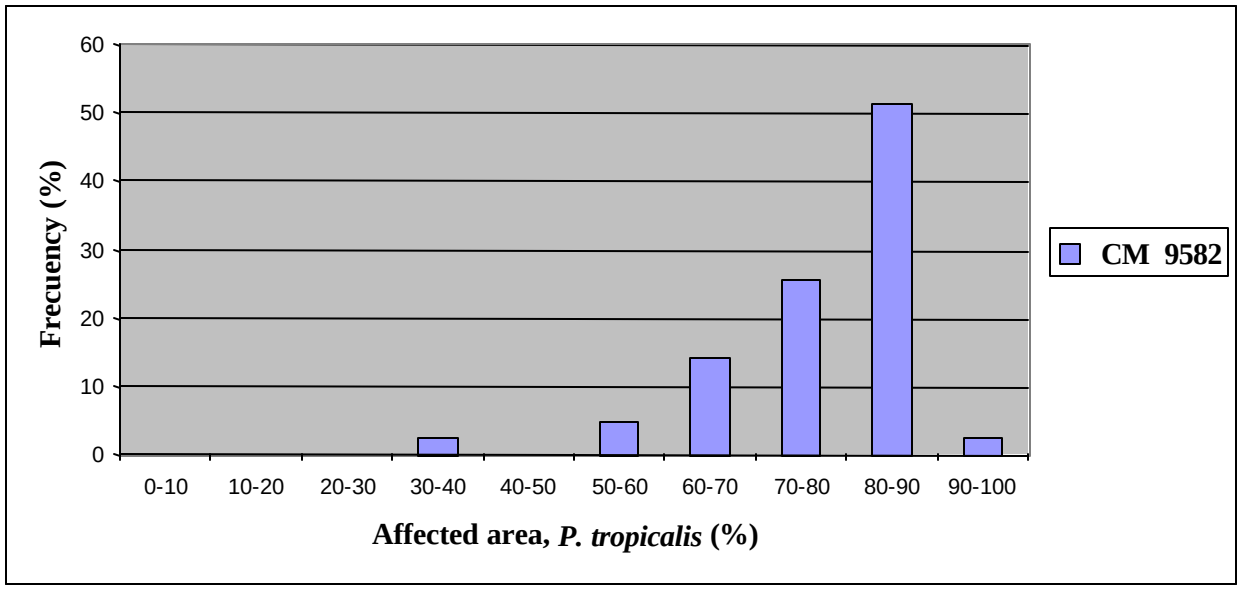

Figure 7.20. Distribution of the frequency of genotypes from the cassava CM 9582 population according to the percentage of area affected by the root rot fungus *Phytophthora tropicalis*.

For the K family across 2000 and 2001, the 10 genotypes with the highest intermediate resistance to *P. tropicalis* had values that ranged between 28% and 47%; and the 10 most susceptible genotypes averaged between 63% and 77%. For the CM 9582 population, the figures were, respectively, 35% and 69% and 84% and 88% (Table 7.19).

Table 7.19. Phenotypic evaluation of two cassava populations segregating for resistance to root rot (RR) caused by the fungus *Phytophthora tropicalis*.

Population or genotype	Reaction to RR: Year 2000	Reaction to RR: Year 2001	Reaction to RR: Average	Population or genotype	Reaction to RR: Year 2001	Reaction to RR: CV (%)
	Family K				CM 9582	
			Intermediate (I) to resistant (R):			
K 19	29.7	27.1	28.4	136	35.2	21.3
K 110	34.2	42.1	38.2	148	49.6	57.3
K 88	54.4	26.0	40.2	150	54.9	25.6
K 98	54.8	28.8	41.8	133	60.3	20.5
K 69	44.2	41.8	43.0	151	61.2	21.0
K 114	32.8	56.6	44.7	121	61.6	16.5
K 79	36.3	53.6	44.9	71	66.7	6.9
K 66	53.2	37.8	45.5	115	67.8	6.2
K 30	55.9	35.8	45.9	140	68.9	13.3
K 81	22.8	70.5	46.7	47	69.1	14.7
Average	41.8	42.0	41.9		59.5	20.3
Correlation between years			-0.67			
			Susceptible (S):			
K 9	60.2	67.3	63.8	42	84.4	5.4
K 57	61.6	65.9	63.8	68	84.7	8.4
K 92	72.7	57.3	65.0	172	85.2	7.5
K 148	62.7	69.4	66.0	153	85.4	13.5
K 39	37.1	95.2	66.1	62	85.9	8.9
K 35	64.9	69.0	66.9	45	86.2	4.4
K 6	69.2	65.9	67.5	52	86.2	9.7
K 122	65.9	71.0	68.5	163	87.5	6.5
K 145	81.6	69.4	75.5	78	87.9	13.2
K 64	67.3	87.2	77.3	91	88.0	5.3
Average	64.3	71.7	68.0		86.3	8.3
Correlation			-0.66			
			General			
Average	53.6	56.0	54.8		76.0	
Correlation			-0.15			
			Parents			
M Nga 2	66.3	56.7	61.5	M CR 81	--	
CM 2177-2	69.6	83.9	76.7	M Bra 1045	46.1	19.6
			Checks			
M Bra 1045 (R)	11.6	51.5	31.5	M Col 2066 (S)	70.9	11.6
M Col 2066 (S)	70.5	86.2	78.3	M Nga 2 (S)	55.4	19.1
				CM 2177-2 (S)	68.3	7.4

Of the 10 intermediately resistant genotypes from the K family, six (K19, K88, K98, K69, K66, and K30) presented very low intermediate resistance during 2000, increasing toward the end of the year. The other four genotypes (K81, K79, K110, and K114) had higher intermediate resistance in 2000 than in 2001.

Of the 10 K family genotypes showing susceptibility in 2001, seven (K9, K57, K148, K39, K35, K122, and K64) were less susceptible in 2000 than in 2001, with the other three being more susceptible.

On average, the 10 intermediately resistant genotypes from the K family were more resistant than the 10 intermediately resistant genotypes from the CM 9582 population. Likewise, on average, the 10 most susceptible genotypes of the CM 9582 population were more susceptible than the 10 susceptible genotypes of the K family.

The coefficient of variation calculated for the CM 9582 population was 12.6%, indicating that the study was reliable with a margin of relatively low experimental error.

For genotypes from the K family with intermediate resistance, the distribution of frequency of genotypes against area infected by *P. tropicalis* presented a curve similar to that of a normal distribution (Figure 7.19). In contrast, for the most susceptible genotypes from the CM 9582 population, the curve was rising (Figure 7.20), with M Bra 1045 showing 56.3% of area infected.

The correlation between root length and area infected was –0.30 for the CM 9582 population, indicating that the longer the root, the less disease found.

Table 7.20 shows the results of the single-marker regression analysis of percentage of infected area in roots inoculated in the laboratory. Markers defined eight QTLs located on linkage groups C, H, J, N, Q, and V (Table 7.20). The QTLs explain between 1.3 and 9% of the variance, the most significant QTL being no. 7, located in linkage group V (chromosome no. 22) of the female-derived framework map.

Table 7.20. QTLs explaining the highest values of variance for resistance in cassava, as described by the percentage of root area infected. Values in bold are significant at $P = 0.05$.

Linkage group (female map)	Markers (position in cM)[a]	F[b]	V[c] (%)	P[d]	QTL no.
C (3)	RGY172	0.029	5.4	<0.0500	1
H (8)	SSRY178	0.315	1.3	<0.0500	2
J (10)	CDY76	0.163	4.0	<0.0500	3
	K2a	0.040	8.6	<0.0500	4
N (14)	SSRY13	0.078	4.2	<0.0500	5
Q (17)	SSRY911	0.047	5.7	<0.0500	6
V (22)	NS911	0.007	9.0	0.0070	7
	GY153	0.049	4.5	<0.0500	8

a. Distance from the first marker noted (o).
b. *F* statistics from analysis of variance.
c. Percentage of variance explained (from r^2 coefficient of regression).
d. Probability of *F* statistic.

Discussion and Conclusions

No reports exist on the genetic basis of resistance to root rot caused by *P. tropicalis* in cassava. Hence, this resistance was evaluated phenotypically in two populations: K family and CM 9582.

K family

Some of the genotypes evaluated from the K family expressed intermediate resistance to *P. tropicalis*, with some presenting intermediate resistance in 2000 but susceptibility in 2001. The opposite also occurred, where some susceptible genotypes in 2000 presented intermediate resistance in 2001.

Such changes may have been triggered by changes in the soil, environmental conditions, or use of chemical products (e.g., fertilizers). These factors indirectly affect partial resistance to pathogens—as corroborated by a study on the partial resistance of maize to *Puccinia sorghi*—and affect QTL expression (Lübberstedt et al. 1998).

Other factors may include the long vegetative cycle, vegetative propagation without quality control of planting stakes, and changes in populations of microorganisms (either beneficial or detrimental) in the rhizosphere and roots. Variability in resistance across years may indicate a polygenic nature of the K family, although the environment usually influences phenotypical expression, generating variation. It is important to note that certain genotypes of the K family with intermediate resistance in 2000 continued expressing it in 2001.

Although both parents of the K family are susceptible to *P. tropicalis*, a group of genotypes from this family showed intermediate resistance. This indicates that the parents are heterozygotes (Fregene et al. 1997) and that they both have resistance genes.

CM 9582 population

The CM 9582 population is obtained by crossing M Bra 1045 with M CR 81. In previous studies, M Bra 1045 has shown resistance to *P. tropicalis*, but in this study, it is susceptible, probably because of changes in environmental factors, as explained above. The genetic base of M Bra 1045 can be assumed to be polygenic, and to have epistasis in this crossing.

The two populations

On comparing the intermediate resistance presented by the K family and CM 9582 population, we found that the CM 9582 population had few genotypes with intermediate resistance to *P. tropicalis*. That is, the 10 most resistant genotypes of the K family had a higher degree of resistance than did the 10 most resistant genotypes of the CM 9582 population. The differences probably lie in the genetic crossings between the parents, which differ for the two populations.

Although the populations differed in their genetic base of resistance to *Phytophthora*, the levels of resistance observed were not sufficiently high to warrant use in genetic improvement programs. Hence, identifying new parents and developing new populations are desirable.

QTLs

Results show that resistance to *Phytophthora* root rot is polygenic in the K family. Results also suggest that the parameters measured for resistance are different and may represent different components of resistance. The occurrence of individuals more resistant than the two parents and the detection of QTLs associated with molecular markers from the female-derived map show that resistance alleles coming from both parents contribute to resistance in the progenies (transgressive segregation). Such characteristics are well known in heterozygous species and are useful for combining resistance genetic factors in the same cultivar (Jorge et al. 2001).

Genotypes classified as resistant in 2000 and susceptible in 2001, and vice versa, can be explained by the effect of environmental factors on the biochemical composition of inoculated cassava roots. Such a hypothesis, however, has to be proved.

Future research, ideally, should include:

- Inoculation of each root with a negative control and the pathogen, thereby reducing the probability of evaluating false positives.
- Use of roots without frogskin disease and with diameters measuring 4 to 7 cm.
- Study of factors influencing the expression of resistance.
- Evaluation of roots from different localities, such as Quindío and Cauca.
- Study of *Phytophthora* pathogenesis in cassava roots and resistance mechanisms.

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Activity 7.13. Super Elongation Disease: Development of hot water treated of cassava cuttings in the greenhouse at CIAT and in the field on the Colombian North Coast and Llanos Orientales.

Greenhouse CIAT

As series of control practices for Super Elongation Disease (SED, causal agent *Sphaceloma manihoticola)* were evaluated in a greenhouse at CIAT. All treatments were applied to two varieties 'Brasilera' (M Col 2737) and 'La Reina' (CM 6740-7). The treatments were:

Treatments

1. Thermotherapy, stem cuttings immersed in a water bath at 49°C during 49 min.
2. Stem cuttings immersed for 5 min in Score® (difenoconazole, 2.5 cc/L of the commercial product)
3. Stakes immersed for 5 min in Kocide® (copper hydroxide, 5g/L of the commercial product)
4. Stakes immersed for 5 min in water
5. Untreated stakes of the variety M Tai 8 was used as a susceptible control.

The experimental design was a randomized complete block design, with seven replicates and 20 plants per treatment for each variety. Each plant was protected with a plastic cover to avoid contamination by spores between the treatments.

Table 7.21. Effect of stake treatments on Super Elongation Disease.

Treatment	SED	
	Germination[a]	AUDPC[b]
CM 6740-7		
Thermotherapy	80	7.0
Score®	95	15.1
Kocide®	85	4.4
Water	85	24.5
M Tai 8	95	0
M Col 2737		
Thermotherapy	100	11.1
Score®	100	19.6
Kocide®	85	7.6
Water	100	20.1
M Tai 8	100	0
M Col 2737 and CM 6740-7		
Thermotherapy	90	10.5 cc
Score®	97	18.3 b
Kocide®	85	6.3 d
Water	92	22.3 a
M Tai 8	97	0 e

[a]Percentage of germination, number of germinated plants.
[b]Average of the Area Under the Disease Progress Curve.
[c]Duncan's multiple range test, alpha <= 0.05.

The highest infections (average AUDPC of 22,3) occurred in the varieties M Col 2737 and Cm 6740-7 treated with water (Table 7.21). This confirms that the stem cuttings, obtained in the field, were highly infected by SED. The highly susceptible variety M Tai 8 did not show any disease symptoms, which indicates that cross contamination did not occur.

Kocide®, a protectant in the case of stem cuttings, provided the best control with an average AUDPC of 6,3. We suggest that foliar applications during the periods when inoculum pressure is great could provide better control. The thermotherapy The AUDPC of 10,5 in thermotherapy treatments was similar to that of water only. Hence we suggest that higher temperatures and longer exposures be tested.

Stem cuttings treated with Score®, which is known to be systemic, had an AUDPC of 19,6. This is a low level of control and for future experiments the immersion time will be prolonged and foliar applications will be made. At the moment in the Atlantic Coast (Sincelejo, Sucre) stem treatment and foliar applications of Score® (2.5 cc/L) and Kocide® (5 g/L) are being tested on susceptible varieties. Stem cuttings were also treated before planting using the

same doses of Score®. This fungicide was more effective than Kocide. Foliar applications appear to be more effective then treatment of stem cuttings. Leave rapidly absorb the product, which affects the subcuticular growth the hyphae of *Sphaceloma manihoticola*. Although the mode of action of the Score® is both has protective and curative, applications should be initiated when the first symptoms of the disease appears (label information, Score®).

Score®® and Kocide® do not persist for long on the leaves, whereas other products like Daconil® (chlorothalonil) which have adherents persist for longer periods and can be applied less frequently then Score®® and Kocide®. This product and others will be tested to optimize disease control.

Field evaluations at the Colombian North Coast

Field tolerance to SED, Cassava Bacterial Blight (CBB) and other disease control practices were evaluated in field trials in the municipality of Sincelejo (Sucre).

The incidence and severity of SED under various treatments (Table 7.22) are currently being evaluated in Sucre with the variety 'Venezolana' (M Ven 25). The regional variety 'Venezolana' was planted with vegetative seed obtained from a farm at Sincelejo, where SED was present. The experimental design was a randomized complete block design, with three replicates and 45 plants per treatment.

Table 7.22. Evaluation of the germination of control practices on SED at the Colombian north coast.

Treatment	Germination (%)[a]
	Sincelejo (Sucre)
Fertilized[b]	
Stake selection	98
Thermotherapy[c]	100
Kocide®, Sistemin®[d]	99
Score®, Sistemin®[e]	100
Control, traditional farmer's practice	100
Without fertilizer	
Stake selection	100
Thermotherapy[c]	100
Kocide®, Sistemin®[d]	100
Score®, Sistemin®[e]	100
Control, traditional farmer's practice	100

[a]Percentage of germination, number of germinated plants.
[b]Sucre: 15-15-15 (NPK) 300 kg/ha.
[c]Stakes immersed in water heated over a wood fire to 49°C for 49 min.
[d]Stakes immersed for 5 min. in Kocide® (copper hydroxide, 5 g/L) and Sistemin® (dimethoato, 3cc/L).
[e]Stakes immersed for 5 min in Score® (difenoconazol, 2.7 cc/L) and Sistemin®. Score® was applied four months after planting.

The following genotypes were planted at Sucre to evaluate resistance to SED and CBB (120 plants/genotype): SM 6758-1, SM 1665-2, CM 6119-5, SM1565-17, CM 4843-1, CM 6754-8,

CM 4919-1, SM 1438-2, M Tai 8 and the local variety Venezolana (M Ven 25). Control measures were not applied.

Germination of cassava plants for the control practices did not present significant differences (Table 7.22). The planted genotypes showed germination percentage from 77% for SM 1665-2 and 100% for M Ven 25 (Table 7.23).

Table 7.23. Evaluation the germination of 10 different cassava genotypes planted at Sincelejo (Sucre).

Genotype	Germination (%)
Sincelejo (Sucre)	
SM 1565-17	99
SM 1438-2	97
CM 4919-1	87
CM 6119-5	86
SM 1665-2	77
CM 6754-8	98
CM 4843-1	74
CM 6758-1	92
M Ven 25	100
M Tai 8	92

Activity 7.14 Detection of a Phytoplasma Associated with Frogskin Disease in Cassava (Manihot esculenta Crantz) in Colombia

Introduction

Frogskin disease (FSD) was first reported in 1971, in the Department of Cauca, southern Colombia, apparently originating from the Amazon region of either Brazil or Colombia (Pineda and Lozano 1981). The disease has since spread throughout Colombia (Atlantic Coast, and Departments of Cauca, Valle de Cauca, Vaupés, and Putumayo), Venezuela (States of Amazonas, Aragua, Barinas, Cojedes, Monagas, and Portuguesa) (Chaparro-Martínez and Trujillo-Pinto 2001), and Brazil.

Frogskin disease directly affects root production, causing yield losses of 90% or more. Although symptoms vary according to temperature and genotype, the roots become thin and woody, and starch content is very low. The causal agent has not been identified, although research so far suggests that FSD may have a viral etiology and may be transmitted by an aerial vector.

Frogskin disease can be controlled by using tolerant varieties, healthy vegetative planting materials, and adequate plant health management.

Materials and methods

Plant tissue

Several molecular and microscopy staining techniques were applied to detect phytoplasmas in plant tissues from cassava (*Manihot esculenta,* 10 samples), periwinkle (*Catharanthus roseus,* 4 samples), and naranjilla or *lulo* (*Solanum quitoense,* 2 samples). Vegetative tissues from the following FSD-infected cassava varieties were used: CM 849-1, SM 1219-9, Parrita, and M Bra 383, all harvested at Jamundí, Valle de Cauca, Colombia. The plants used were about 12 months old. The roots were severely infected by FSD. The leaves and flowers did not show visible symptoms caused by phytoplasmas (such as witches' broom) or viruses. Samples of healthy 'Secundina', obtained by *in vitro* culture of meristem tips, were used as negative control. Infected plants from plots at CIAT (Palmira) were also included in the analysis.

Microscopy

Two staining methods were used: DAPI (4,6 diamidine 2-phenylindole), which stains the phloem (Sinclair et al. 1989); and Dienes' stain, which metabolizes and produces a blue color (Deeley et al. 1979).

DNA extraction

Total DNA was extracted as described by Gilbertson and Dellaporta (1983) from samples of each of the following tissues: roots, stems, petioles, leaf midribs, and flowers of FSD-infected and healthy cassava plants. DNA was also extracted from the leaves of naranjilla and periwinkle, infected by phytoplasmas. DNA was diluted in sterilized deionized water to a final concentration of 20 ng/µL.

Direct and nested PCR. DNA samples were amplified in a nested PCR. For the first amplification, we used the primer pairs P1/P7 or R16mF2/R16mR1 (Table 7.24) under the following conditions: 120 ng of diluted DNA, 1X buffer, 3 mM $MgCl_2$, 0.8 mM dNTPs, 0.1 µM of each primer, and 1U *Taq* polymerase. Thirty-five cycles were conducted in a PTC–100 thermocycler (Programmable Thermal Controller, MJ Research, Inc., Watertown, MA) as follows: 1 min (2 min for the first cycle) denaturation step at 94 C, annealing for 2 min at 55 C, and primer extension for 3 min (10 min in final cycle) at 72 C. PCR products were diluted at 1:10 with sterilized deionized water. For the nested PCR, we used 2 µL of diluted PCR to amplify with the primer pair R16F2n/R16R2 as described above, but using an annealing temperature of 50 C. PCR products were analyzed by electrophoresis on 1.2% agarose gels and photographed, using an Eagle Eye II I image analyzer (Stratagene, La Jolla, CA).

Table 7.24. Primers used for PCR amplification and sequencing of 16S rRNA genes of plant pathogenic phytoplasmas.

Primer	Sequence 5' - 3'	Reference
R16R2	TGACGGGCGGTGTGTACACCCG	Gundersen and Lee (1996)
R16mF2	CATGCAAGTCGAACGGA	Gundersen and Lee (1996)
R16mR1	CTTAACCCCAATCATCGAC	Gundersen and Lee (1996)
R16F2n	GAAACGGCGGTGTGTACAAACCCCG	Gundersen and Lee (1996)
P1	AAGAGTTTGATCCTGGCTCAGGATT	Deng and Hiruki (1991)
P7 (23S)	CGTCCTTCATCGGCTCTT	Smart et al. (1996)

RFLP analyses
The nested-PCR products of the controls, and the 16S rDNA sequences of cassava, periwinkle, and naranjilla were amplified with primer pair R16F2n/R2. A 5-µL aliquot of each PCR product (1.2 kb) was digested with each of the restriction endonucleases *Alu*I and *Rsa*I according to manufacturer's instructions (Promega, Madison, WI). The restriction products were then analyzed on a 2% agarose, visualized, and saved in a gel documentation system (Eagle Eye II, Strategene). The restricted-DNA patterns of infected cassava, periwinkle, and naranjilla were compared with the RFLP patterns produced by the control strains.

Cloning, transformation, and sequencing of DNA
Six PCR products were sequenced directly, using a DNA-sequencing kit from Applied Biosystems, with 3 µL water, 1 µL primer, 4 µL mix from kit, and 1 µL DNA. The PCR products were purified, using the QIAquick PCR Purification Kit (QIAGEN), ligated in pGEM-T Easy vector, which was introduced into the *Escherichia coli* strain DH5-α by electroporation at 2.4 kV/cm^2. Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media. Plasmids were extracted with a Plasmid Miniprep System Kit (Gibco-BRL). Positive inserts were observed by plasmid restriction with *Eco*RI and electrophoresis in 1.5% agarose gel. Different-sized fragments were selected for sequencing by automated dideoxy sequencing (ABI Prism 377-96 DNA Sequencer), using a DNA-sequencing kit from Applied Biosystems, with 3 µL water, 1 µL primer, 4 µL mix from kit, and 1 µL DNA. Sequences were analyzed with Sequencer 4.1 software and matched by nucleotide, using the Blastn tool in GenBank (www.ncbi.nlm.nih.gov).

Results and discussion

CIAT's Cassava Pathology programmed a series of activities aimed at identifying the possible causal agent of FSD in cassava. The principal advances are summarized below.

For many crops, the causal agents of similar diseases were considered to be viruses. However, over the last 20 to 25 years, the causal agents were found to be phytoplasmas. For example, lethal yellowing disease in the coconut palm was reported by Nutman and Roberts (1955) as being viral, whereas Beakbane et al. (1972), Heinze et al. (1972), Plavsic-Banjac et al. (1972), and (Mariau et al. 2002) all identified the causal agent as being a phytoplasma.

In this study, we present evidence that FSD is associated to a phytoplasma and that, by applying molecular tools and microscopy, we successfully detected phytoplasmas in FSD-infected cassava roots, leaf midribs, petioles, and peduncles.

The specific primers R16mF2/R16mR1 and R16F2n/R16R2 were successfully used in a nested-PCR assay to detect and confirm that phytoplasmas were associated with FSD.

To detect and subsequently classify the phytoplasmas, two pairs of universal primers (P1/P7 and R16F2n/R2) were used to amplify the 16S rDNA gene. A 1.2-kb fragment was amplified from all samples, including infected roots (Figure 7.21). This fragment was present only in

samples collected from plants showing visible external symptoms in the roots. Direct PCR, using the primers R16mF2 and R16mR1 also detected phytoplasmas.

The presence of phytoplasmas in roots, stems, petioles, leaf midribs, and flowers was confirmed by DAPI and Dienes' stain by microscopy (Figures 7.22 and 7.23).

Sequence analysis of the cloned fragment (Figure 7.24) revealed that the cassava phytoplasma was similar to the chinaberry yellows phytoplasma (GenBank acc. no. AF495657, 16SrXIII Mexican periwinkle virescence group) and cirsium white leaf phytoplasma (GenBank acc. no. AF373106, 16SrIII X-disease group), both with a sequence homology of 100% and 99% in two partial fragments with a total of 1.01 kb (Table 7.25). The sequence length was 1202 bp (Table 7.26).

According to the RFLP patterns with *Rsa*I, the cassava phytoplasma was similar to that for naranjilla, whereas that for periwinkle was different again. *Alu*I did not highlight differences among the samples (Figure 7.25). Future research will involve evaluation with another group of enzymes, and sequence analysis will be carried out to classify the phytoplasmas.

We have already started studies on the transmission of the causal agent of FSD. Remission experiments, using chlortetracycline, with cassava, periwinkle, and poinsettia are being conducted, and we will need to determine the role of phytoplasmas in this destructive disease.

This is the first report of phytoplasmas being associated with FSD in cassava. Future research topics will include the development of molecular detection methods, vector identification, and classification of phytoplasmas associated with FSD. The design of novel approaches to achieve effective control will remain a constant goal.

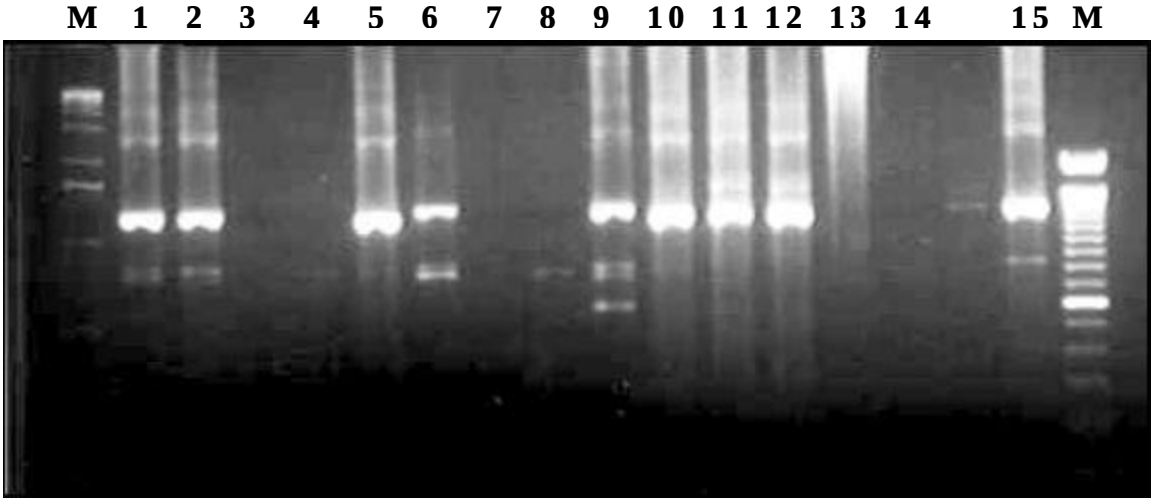
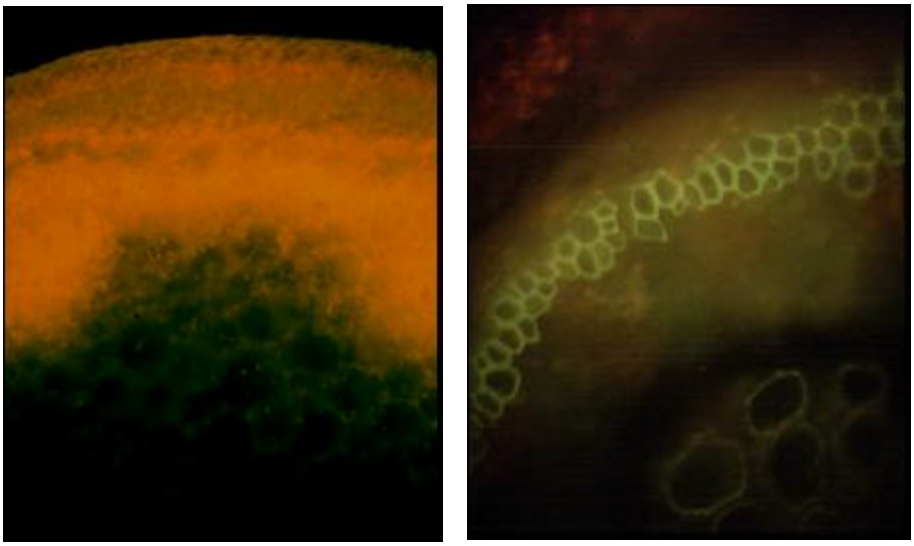
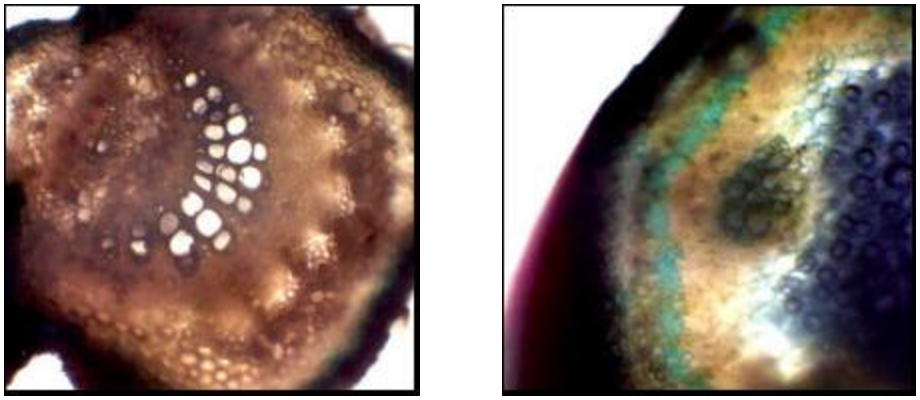


Figure 7.21. Nested PCR of infected and healthy plant tissues, using primers R16MF2, R16MR1/R16F2N, R16FR2. Lanes 1 and 2 = infected cassava roots (pulp); lanes 3 and 4 = leaves and shoot from healthy cassava plants; lane 5 = stem tissue from an infected cassava plant; lane 6 = petiole from an infected cassava plant; lanes 7 and 8 = peel from infected cassava roots; lanes 9 and 10 = infected cassava roots; lanes 11 and 12 = leaf tissue from naranjilla and periwinkle, respectively; lane 13 = degraded DNA from periwinkle; lane 14 = negative control without DNA; lane 15 = positive control; lane M = bp ladder.



A **B**

Figure 7.22. DAPI stain of healthy (A) and infected (B) cassava leaf tissue.



A **B**

Figure 7.23. Dienes' stain of healthy (A) and infected (B) cassava leaf tissue.

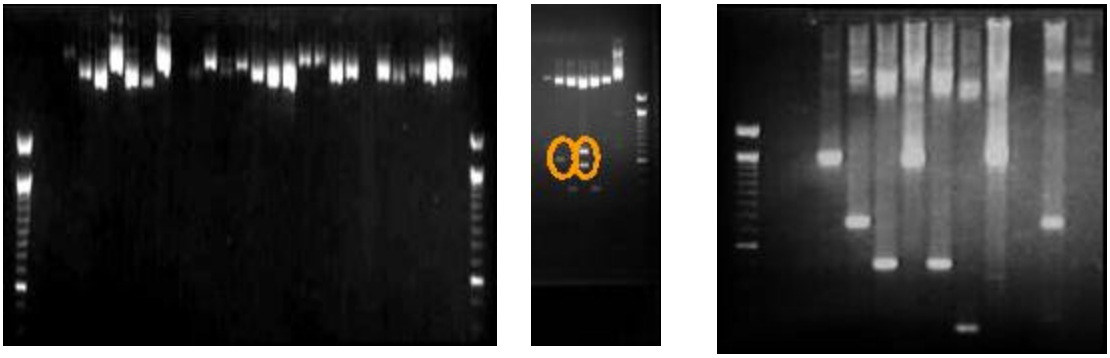


Figure 7.24. Cloning and restriction with *Eco*RI: (A) plasmids; (B) their restriction; and (C) PCR to confirm presence of inserts in the plasmids.

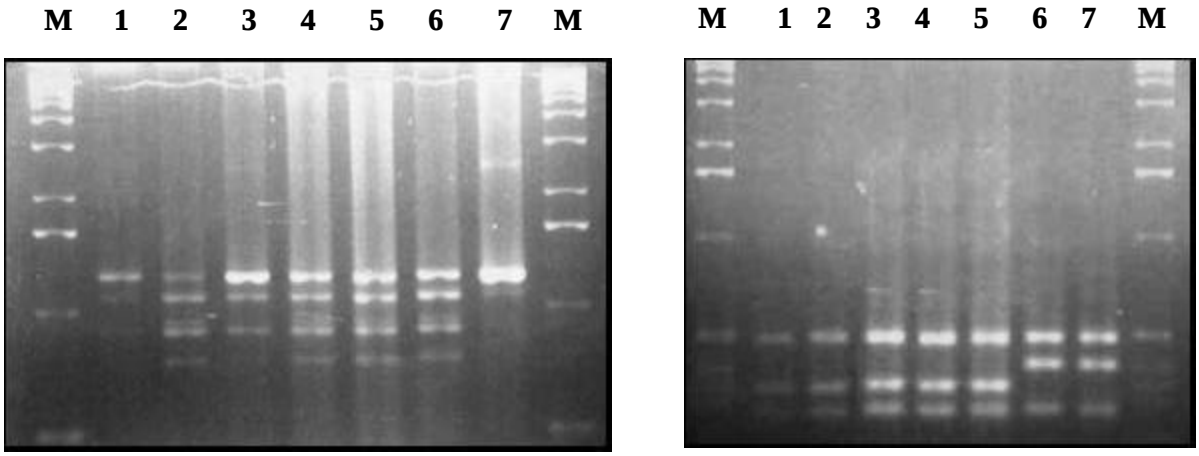


Figure 7.25. RFLP patterns obtained: (A) *Alu*I; (B) *Rsa*I. Lanes 1-3 = cassava; lanes 4 and 5 = naranjilla; lanes 6 and 7 = periwinkle; lane M = bp ladder.

Table 7.25. Homology found between DNA obtained from cassava infected by FSD by nested PCR and phytoplasma sequences reported in GenBank.

Matching in GenBank	GenBank number	Sense	Homology score (bits)	Probability of higher homology	Homologued fragment (bp)	Identities	
						Absolute	(%)
Cirsium white leaf phytoplasma rRNA operon B	AF373106.1	5'- 3'	1084	0.04	546	546	100.0
		3'- 5'	856	0.0	465	459	98.7
Chinaberry yellows phytoplasma 16S rRNA gene	AF495657.1	5'- 3'	1084	0.0	546	546	100.0
		3'- 5'	872	0.0	465	461	99.1
Chayote witches' broom phytoplasma ChWBIII strain 16S rRNA gene, 16S–23S rRNA intergenic	AF147707	5'- 3'	1076	0.0	546	545	99.8
		3'- 5'	856	0.0	465	459	98.7
Poinsettia branch-inducing phytoplasma rRNA operon B	AF190223	5'- 3'	1068	0.0	546	544	99.6
		3'- 5'	856	0.0	465	459	98.7
Gaillardia phyllody phytoplasma 16S rRNA gene	AY049029	5'- 3'	1060	0.0	542	540	99.6
		3'- 5'	872	0.0	465	461	99.1
Dandelion virescence phytoplasma rRNA operon B	AF370120.1	5'- 3'	1045	0.0	546	541	99.1
		3'- 5'	864	0.0	465	460	98.9

Table 7.26. Sequences of a phytoplasma obtained from cassava infected by frogskin disease.

Identification	Size (bases)	Sense	Sequence
PCR-6RF Phytoplasma	546	Forward	TTGAAGGTATGCTTAAGGAGGGGCTTGCGACACATTAGTTAGTTGGCAGGGTAAAGGCCT ACCAAGACTATGATGTGTAGCTGGACTGAGAGGTTGAACAGCCACATTGGGACTGAGAC ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGGCAATGGAGGAAACTCT GACCGAGCAACGCCGCGTGAACGATGAAGTACCTCGGTATGTAAAGTTCTTTTATTAAGG AAGAAAAAAGAGTGGAAAAACTCCCTTGACGGTACTTAATGAATAAGCCCCGGCTAATTAT GTGCCAGCAGCCGCGGTAATACATAAGGGGCGAGCGTTATCCGGAATTATTGGGCGTAA AGGGTGCGTAGGCGGTTTAATAAGTCTATAGTTTAATTTCAGTGCTTAACGCTGTTGTGCT ATAGAAACTGTTTTACTAGAGTGAGTTAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAA ATGCGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGACTTTACTGAC GCTGAGGC
PCR-6RR2 Phytoplasma	593	Reverse	CGAAATGCTGATTCGCGATTACTAGCGATTCCAACTTCATGAAGTCGAGTTGCAGACTTCA ATCCGAACTGAGATTGATTTTGTGAGATTGGCTAAGAACTCGCGTTTCAGCTACTCTTTGT ATCAACCATTGTATCACGTTTGTAGCCCAGATCATAAGGGGCATGATGATTTGACGTAATC CCCACCTTCCTCCAATTTTTCATTGGCAGTCTCGTTAAAGTCCCCATCATTACATGCTGGC AATTAACGACAAGGGTTGCGCTCGTTTTAGGACTTAACCTAACATCTCACGACACGAGCT GACGACAACCATGCACCACCTGTTTTCCTGATAACCTCCATTATATTTCTATAACTTCGCA AGAAAATGTCAAGACCTGGTAAGGKTTTTCGTGTATTCTTCGAAATTAAACAACATGGATC CACCGCTTGTGCGGAGTCCCGTCAATTCCTTTAAGTTTCATACCTTGCGTAACGGNACTA CTCAGGCGGGAGGACTTAATGGTGTTAAACTTTCAANAAACCGGGGTTTACCCGGAACAC YTTAANTACCTCAATTCGGTTTACGGGNGGTKGGGACCTACCCAGGG

Acknowledgements

We thank Francy Baron and Carlos Huertas (both of CORPOICA, Palmira, Colombia) for providing samples of periwinkle and naranjilla infected by phytoplasma, staining samples, and conducting microscopic observations. We also thank Agrovelez S.A. (Jamundí, Valle) for access to field materials.

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Activity 7.15. Evaluating Thermotherapy and Biological Products for Controlling Frogskin Disease in Cassava

Objectives

Frogskin disease (FSD) in cassava is transmitted by planting contaminated stakes. No effective method exists for disinfecting stakes and preventing the disease's dissemination. In the following experiments we aim to develop a methodology that includes products and processes for disinfecting cassava stakes of FSD.

Methodology

Experiment 1

Stakes of the cassava variety Parrita—a Chiroza type for the fresh market—were collected from a highly infected commercial cassava crop at Jamundí (Department of Valle, Colombia). Stakes were selected at harvest time to ensure FSD was present. About 80% of the plants presented symptoms, many very severely. The stakes were planted into pasteurized soil, free of FSD, in pots placed in an isolated greenhouse at CIAT.

Before planting, the cassava stakes were treated with one of the following: thermotherapy; immersion in fresh, whole, cow's milk; and immersion in gliricidia (*Gliricidia sepium*) leaf extract. The methodology for thermotherapy, involving hot water, was based on that developed to control *Phytophthora* species (sp. *tropicalis* and others) and *Xanthomonas axonopodis* pv. *manihotis*. Cow's milk has been reported to denaturalize tomato virus and gliricidia is well known in organic agriculture as a viricide.

Treatments:

1 No treatment

2 *Trichoderma* sp., strain 14PDA-4 (1×10^6 conidia/mL); stakes immersed for 30 min and solution applied to soil at planting time

3 Tachigaren® (hymexazol, granular presentation), 0.75 g/L; stakes immersed for 30 min and solution applied to soil at planting time

4 Whole, pure, fresh, cow's milk; stakes immersed and not rinsed

5 Gliricidia (leaf extract, 100 g/L in 50% alcohol, blended, 1 night fermentation), stakes immersed and solution applied to soil at planting time

6 to 20 Hot water therapy:
- immersion in hot water for 49 min at 49°C (no pretreatment)
- pretreatment for 20 min at 49°C followed by 1, 2, or 3 h at each of 49°C, 51°C, 53°C, 55°C, 57°C

Experiment 2
Three genotypes from Jamundi were used: M Bra 383, CM 849-1, and SM 1219-9. Incidence of FSD was between 10% and 70%, according to genotype. Experimental conditions and characteristics were similar to those of Experiment 1.

Treatments:

A. Stakes planted vertically:
1 Gliricidia (50% ethanol, no filtering), stakes immersed, applications to soil
2 Milk (pure), stakes immersed and not rinsed
3 Stakes immersed in water
4 No treatment

B. Stakes planted horizontally:
1 Control
2 to 17 Hot water therapy:
- immersion in hot water for 49 min at 49°C (no pretreatment)
- pretreatment for 20 min at 49°C followed by 1, 2, or 3 h at each of 49°C, 51°C, 53°C, 55°C, 57°C

Experiment 3
See Experiment 2 for the genotypes and experimental design used. Cassava stakes were prepared either with or without a longitudinal perforation that extended from the top of the stake to the center of the medula, using a small drill. After treatment, the top of the stakes were covered with paraffin.

Treatments:

1 and 2 Chlortetracycline (100 ppm or 1000 ppm), stakes immersed and applications (at 1000 ppm) to foliage on a weekly basis

3 and 4 Chloramphenicol (100 ppm or 1000 ppm), stakes immersed and applications (at 1000 ppm) to foliage on a weekly basis

5 Chlortetracycline (1000 ppm), stakes immersed, and alternating weekly foliar applications with either chloramphenicol or chlortetracycline at 1000 ppm

Results and discussion

Although the experiments are still continuing, we found that germination rates of cassava after the stakes were treated with milk and gliricidia improved by rinsing the stakes (milk) or filtering the extract (gliricidia).

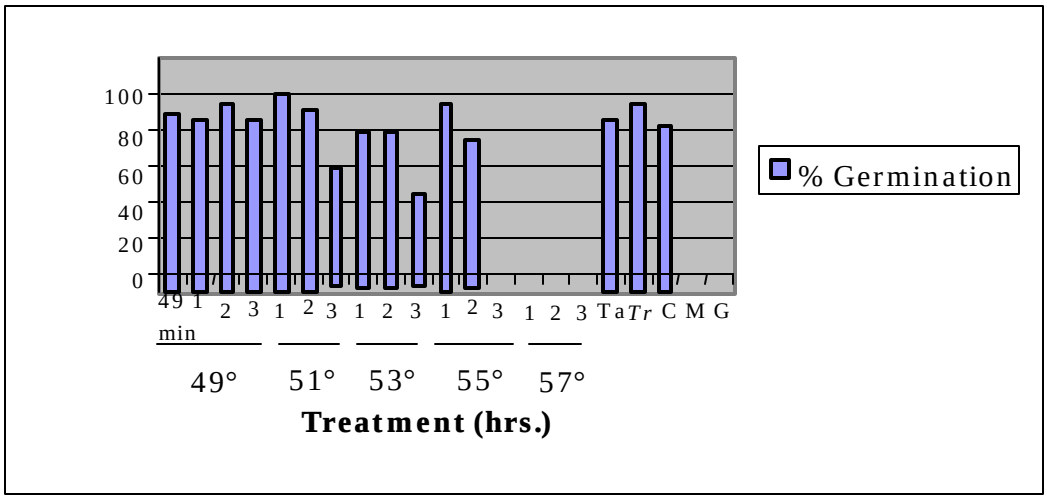

Figure 7.26. Effect of hot water treatment, Tachigaren® (Ta), *Trichoderma* (Tr) isolate 14PDA-4, milk (M), and gliricidia (G) on germination rates of stakes from the cassava variety Parrita infected with FSD.

Stakes of the variety Parrita treated with hot water for 3 h at 55°C and for any time at 57°C did not germinate (Figure 7.26). Germination rates after the other temperatures (49°C, 51°C, and 53°C) were highly acceptable. Germination rates after 1 or 2 h at 55°C were 88% and 78%, respectively. These treatments included a pretreatment of 20 min at 49°C, 24 h before the main treatment.

Hot water treatment of stakes from M Bra 383, CM 849-1, and SM 1219-9 at 53°C (including pretreatment) improved germination (average of 80.6%), compared with no treatment (75.6%). Germination after pretreatment for 10, 20, or 30 min and followed by a main treatment at 53°C for 1 h was, respectively, 91.7%, 77.8%, and 86.1%. Where no pretreatment was given, germination was 66.7%, thus highlighting the importance of applying a pretreatment to improve germination.

Activity 7.16. Promoting the adoption of CIAT cassava genotypes among indigenous groups of Mitú, Department of Vaupés, Colombia

CIAT genotypes CM 2772-3, ICA Catumare, M Bra 1044, and M Bra 97 were adopted by women farmers of the Tukano indigenous group in Mitú, Department of Vaupés, Colombia. Women from five indigenous communities planted these varieties, together with native varieties, in several of their *chagras* (small farming plots). They followed traditional planting arrangements, which consisted of 3 to 30 or more varieties associated with other crops. Some CIAT varieties were also being grown in *chagras* of communities outside the project's area of influence. So far, their performance has been acceptable, with yields similar to those of native ones, and the quality of their starch and flour is appropriate for preparing traditional foods.

Activity 7.17. Collecting cassava landraces around Mitú

A native cassava collection was conformed and planted at departmental farm of Vaupés (Mitú), with 23 varieties collected from chagras in different Tukanoan indigenous communities settled close the Vaupés river and the road Mitú – Monfort (Table 7.27).

Table 7.27. Native cassava varieties collected in different Tukanoan communities in Mitú (Vaupés) and planted at Vaupés departmental farm (Mitú).

Variety	Tukanoan name	Root pulp color	Variety	Tukanoan name	Root pulp color
Castaña	Castaña ducú	Yellow	Yuca de Chicharra	Ñairoa ducú	White
Gallineta	Ajá ducú	Yellow	Yuca de Cuya	Bajato ducú	White
Inayá	Isquí ducú	White	Yuca de güío	Piró ducú	Yellow
Mirití	Neé ducú	Yellow	Yuca de maíz	Jocó ducú	White
Patabá	Ñumú ducú	White	Yuca de Mico	Asque ducú	Yellow
Siringa	Waso ducú	White	Yuca de Mojarra	Varía ducú	Cream
Tucunaré	Buú ducú	Yelow	Yuca de paca	Semé ducú	Cream
Vapu	Váspu ducú	Cream	Yuca de Pato	Patu ducú	Cream
Wasaí	Mispï ducú	Yellow	Yuca de Puño	Buú ducú	White
Wansoco	Utañimí ducú	White	Yuca de uva	Usé ducú	White
Yuca de Abejorro	Veranu ducú	White	Yuca roja	Soarí ducú	White
Yuca de algodón	Busá ducú	Yellow			

Activity 7.18. Multiplying promising cassava genotypes to ensure sufficient planting material for both greenhouse and field experiments

A total of 159 promising cassava genotypes are being propagated in a farm located in Rozo, Palmira (Department of Valle del Cauca, Colombia) for greenhouse experiments on varietal resistance, genetic studies, and disease management.

Activity 7.19. Using meristem culture to clean cassava cuttings of frogskin disease

Table 7.28 shows the number of cassava genotypes being cleaned of FSD for use by CIAT's Cassava Pathology Program for experiments on varietal resistance, genetic studies, and disease management. By thermotherapy, meristem culture, and grafting with 'Secundina', 33 genotypes have been cleaned. A total of 93 genotypes are kept *in vitro*, with 8 clones (plants) each one, corresponding to 744 clones.

Table 7.28. Cassava genotypes cleaned of frogskin disease for use in different cassava pathology research projects at CIAT.

Step	Genotypes in process	Clones (no.)	Results
Thermotherapy	19		
Meristem propagation in vitro	93	8	744 clones in 4E, 17N, and 8S culture media
ELISA assay	26	100	CCMV = 3 positive clones CsXV = 6 positive clones
In screenhouse, no grafting	5	5	
Grafting with 'Secundina'	33	87	FSD = 2 positive clones

The 'Secundina' genotype has been propagated *in vitro* to obtain disease-free plants for use as an FSD indicator in assays searching for the virus vector and disease management. A total of 84 plants, 40 in the screenhouse and 44 *in vitro*, were obtained last month.

Activity 7.20. Training farmers, technicians, and extension agents in participatory research, cassava management, oil-palm cultivation, and disease control strategies

Courses

- Fundamentals in molecular biology for plant pathologists
  For ASCOLFI (Colombian Association of Plant Pathology and Related Sciences), September 2001

- Modern systems of cassava production and processing in Colombia: cassava disease management
  El Espinal, 20-22 November 2001

- Cassava production: integrated disease management
  Three courses for 137 participants
  Venezuelan municipalities of El Tigre (Anzoátegui), San Carlos (Cojedes), and Maracaibo (Zulia), 4-15 May 2002

- Modern production, processing, and utilization systems
  For Technicians and farmers. CLAYUCA, 25-28 June 2002

- Integrated management of cassava pests and diseases
  For Master Science students from Escuela Politécnica del Ejército, ESPE, Ecuador
  10-12 September 2002

Seminars

- Management of major cassava diseases in the North Coast, with emphasis on superelongation disease
  Sincelejo, 19 November 2001

- Molecular biology techniques applied to crop pathogen identification and characterization. Seminar given during a workshop for an international course on "Tropical hortifruticulture with emphasis on organic production and biological management"
  For 22 participants from Latin America, with two participants from the Cabildo de Guambía (Silvia, Cauca)
  CIAT, 15-16 November 2001

- Advances in cassava pathology research
  For 30 students from the Universidad de Caldas
  Manizales, 20 December 2001

- Cassava disease management
  For Alejandro Larios, starch producer from Caicedonia (Valle)
  CIAT, 7 February 2002

- Superelongation disease management in cassava. Seminar given during the I Regional Workshop on Fast Propagation (*In Vitro*) and Genetic Transformation
  32 participants from Brazil, Venezuela, Ecuador, and Colombia
  CIAT, 26 February 2002

- Advances in cassava pathology research
  For 34 students from the Universidad de Caldas
  Manizales, 18 April 2002

- Cassava diseases: diagnosis and control
  For 4 researchers from Haiti
  15 May 2002

- Cassava pathogens
  For 30 bacteriology students from the Universidad del Valle
  Cali, July 2002

- Integrated management of cassava diseases
  For Manuel Naranjo and Jorge Peña, cassava agronomists from Casanare
  5 August 2002

- Advances in the knowledge and management of rose mildews
  For ASOCOLFLORES
  Bogotá, 29 August 2002

- Principal cassava pathogens. Seminar given during the III International Congress of the National College of Bacteriologists (CNB)

Universidad del Valle, Cali, 1-4 November 2002

- Advances in cassava pathology research
  For 32 students from the Universidad de Caldas
  Manizales, 26 September 2002

Training

- Three courses on soil management and integrated pest and disease management
  242 indigenous women farmers of the communities of Cucura, Bocas del Yí, and Macaquiño, Colombia
  October-December 2002

- Establishing Local Agricultural Research Committees
  15 indigenous farmers and local technicians
  April 2002

- Isolating *Sphaceloma manihoticola* and understanding superelongation disease
  Juan Manuel López, Professor of Genetics, Universidad de Sucre, Colombia
  April 2002

- Isolation and inoculation of *Phytophthora* sp. in soybean
  Ana Claudia Gordillo, CORPOICA "La Libertad", Villavicencio
  April 2002

- Isolation and inoculation of *Phytophthora* spp., *Pythium* sp., *Fusarium* spp.
  Alexandra Delgado, Hacienda San José, Palmira
  April-September 2002

- Soilborne pathogens in cassava and sugarcane
  Mariela Becerra, Universidad Francisco de Paula Santander Facultad de Ciencias Agrarias y del Ambiente
  Cúcuta, May-June 2002

- Isolation of *Ralstonia solanacearum* from plantain and banana tissue, soil, weeds, and water in crops affected by *moko*
  Luz Piedad Estrada, ICA—Quindío
  June 2002

- Isolation of *Ralstonia solanacearum* from plantain and banana tissue, soil, weeds, and water in crops affected by *moko*
  Ana Lucía Gaviria and Yaneth Rivera, Universidad del Quindío, Armenia
  June 2002

- Isolation of *Ralstonia solanacearum* from plantain and banana tissue, soil, weeds, and water in crops affected by *moko*, and management of *moko* by disinfection of soil and tools

Rosinelly Pérez, Especial, La Tebaida, Quindío
July 2002

- Isolation of *Ralstonia solanacearum* from plantain and banana tissue and soil in crops affected by *moko*
  Carlos Aníbal Montoya, ICA—Palmira
  16 July 2002

- Cassava disease management
  Norman Pérez, Chemonics, Putumayo
  18 July 2002

- Molecular and traditional characterization of *Ralstonia solanacearum*.
  Abraham Oleas, Ecuador
  16-17 September 2002

- Biological controllers
  César Cano, Perkins
  July-August 2002

- *Phytophthora* spp. culture management
  Alejandro Corredor, Universidad de Caldas, Manizales
  9 August 2002

Publications, Awards, Meetings, and Theses

Publications

Caracterización genética y patogénica en Colombia de *Sphaerotheca pannosa* var. *rosae*, agente causal del mildeo polvoso en rosa.

E Alvarez, JL Claroz, JB Loke, C Echeverri. Fitopatología Colombiana 25(1-2):7-14, 2001.

Control del mildeo polvoso en cultivos de rosa por aplicación de un lixiviado de compost de plátano.

E Alvarez, CX Grajales, J Villegas, GA Llano, JB Loke. Poster presented at the XV Muestra Agroindustrial y Empresarial. Universidad La Gran Colombia, Armenia, Quindío, 1-3 November 2001.

Desarrollo de prácticas ecológicas de manejo de pudrición radical (*Phytophthora* spp.) en yuca (*Manihot esculenta*).

E Alvarez, JB Loke, GA Llano. Paper presented at the XXIII Congreso Nacional de Fitopatología, held by ASCOLFI, Bogotá, 3-6 July 2002.

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Awards

Second place, XV Muestra Agroindustrial y Empresarial, Universidad La Gran Colombia, Armenia, Quindío, 1-3 November 2001, for:

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Nomination for indigenous communities from Vaupés for the 2002 Equator Prize of UNDP for:

Participatory research on the control of Phytophthora root rots in cassava, conservation of native cassava varieties, and agroecosystem sustainability.

E Alvarez, GA Llano.

Meetings attended

XXIII Congreso Nacional de Fitopatología, held by ASCOLFI, Bogotá, 3-6 July 2002.

Perspectivas de la producción ecológica para productos hortifrutícolas, held by Fundación Centro de Investigiación Hortofrutícola de Colombia – CENIHF. And Corporación Autónoma Regional del Cauca CVC. Roldanillo, Valle, 26-27 July 2002.

Bachelor theses presented

Juan Fernando Mejía. 2002. Caracterización molecular y patogénica de aislamientos de *Sphaceloma manihoticola* del sur y centro de Brasil. Universidad Nacional de Colombia—Palmira.

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CORPOICA—Bogotá (Dr Jairo Osorio)
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